ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Volume XLV, Art. 9. Pages 357–436

Editor
Roy Waldo Miner
Associate Editor (Physics and Chemistry)
Joseph S. Fruton

ENERGY RELATIONSHIPS IN ENZYME REACTIONS

BY

Joseph S. Fruton, Eric G. Ball, Max Bergmann, Herman M. Kalckar, Otto Meyerhof, and Carl V. Smythe

NEW YORK
Published by the Academy
November 10, 1944

THE NEW YORK ACADEMY OF SCIENCES
(LYCEUM OF NATURAL HISTORY, 1817–1876)

OFFICERS, 1944

President: DUNCAN A. MACINNES

Vice-Presidents: G. MARSHALL KAY; RALPH H. CHENEY; ANNE ROE; HORTENSE POWDERMAKER; LEWIS G. LONGSWORTH; RAYMOND B. MONTGOMERY

Recording Secretary: MICHAEL HEIDELBERGER

Corresponding Secretary: H. HERBERT JOHNSON

Treasurer: DONALD BELCHER

Librarian: BARNUM BROWN

Editor: ROY WALDO MINER

Elected Councilors:

1942–1944: ERICH M. SCHLAIKJER; LAWRENCE K. FRANK; RAYMUND L. ZWEMER

1943–1945: CHARLES M. BREDER, JR.; HERBERT J. SPINDEN; HAROLD E. VOKES

1944–1946: WALTER H. BUCHER; VICTOR K. LAMER; THEODORE SHEDLOVSKY

OFFICERS OF SECTIONS

GEOLOGY AND MINERALOGY

Chairman: G. MARSHALL KAY. *Secretary:* RALPH J. HOLMES

BIOLOGY

Chairman: RALPH H. CHENEY. *Secretary:* RAYMUND L. ZWEMER

PSYCHOLOGY

Chairman: ANNE ROE. *Secretary:* LIVINGSTON WELCH

ANTHROPOLOGY

Chairman: HORTENSE POWDERMAKER. *Secretary:* GORDON F. EKHOLM

PHYSICS AND CHEMISTRY

Chairman: LEWIS G. LONGSWORTH. *Secretary:* RAYMOND M. FUOSS

OCEANOGRAPHY AND METEOROLOGY

Chairman: RAYMOND B. MONTGOMERY. *Secretary:* JOHN C. ARMSTRONG

The Sections of Geology and Mineralogy, Biology, Psychology, and Anthropology meet in regular session on Monday evenings at 8:00 o'clock from October to May, inclusive. The Sections of Physics and Chemistry, and Oceanography and Meteorology hold two-day conferences at irregular periods. All meetings of the Academy are held at The American Museum of Natural History, Central Park West at 79th Street, New York, N. Y.

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
VOLUME XLV, ART. 9, PAGES 357–436

NOVEMBER 10, 1944

ENERGY RELATIONSHIPS IN ENZYME REACTIONS*

BY

JOSEPH S. FRUTON, ERIC G. BALL, MAX BERGMANN, HERMAN M. KALCKAR, OTTO MEYERHOF, AND CARL V. SMYTHE

CONTENTS

PAGE

INTRODUCTORY REMARKS TO THE CONFERENCE ON ENERGY RELATIONSHIPS IN ENZYME REACTIONS. BY JOSEPH S. FRUTON 359

ENERGY RELATIONSHIPS OF THE OXIDATIVE ENZYMES. BY ERIC G. BALL 363

ENERGY RELATIONSHIPS IN GLYCOLYSIS AND PHOSPHORYLATION. BY OTTO MEYERHOF 377

THE FUNCTION OF PHOSPHATE IN ENZYMATIC SYNTHESES. BY HERMAN M. KALCKAR 395

THE SIGNIFICANCE OF COUPLED REACTIONS FOR THE ENZYMATIC HYDROLYSIS AND SYNTHESIS OF PROTEINS. BY MAX BERGMANN AND JOSEPH S. FRUTON 409

SOME ENZYME REACTIONS OF SULFUR COMPOUNDS AND THEIR POSSIBLE INTERRELATIONSHIP. BY CARL V. SMYTHE 425

---

* This series of papers is the result of a conference on Energy Relationships in Enzyme Reactions held by the Section of Physics and Chemistry of The New York Academy of Sciences, February 11 and 12, 1944.

Publication made possible through a grant from the income of the Conference Publications Revolving Fund.

Copyright 1944

by

The New York Academy of Sciences

INTRODUCTORY REMARKS

By

Joseph S. Fruton

Rockefeller Institute for Medical Research, New York, N. Y.

This discussion on "Energy Relationships in Enzyme Reactions" is another in the series of conferences sponsored by the Section of Physics and Chemistry of the New York Academy of Sciences. The subject of our conference is essentially biochemical, but like all borderline disciplines, biochemistry owes much of its vitality to the nourishment it constantly derives from the older sciences from which it arose. It is appropriate, therefore, that we should welcome to this conference not only confessed biochemists, but also biologists, physiologists, organic chemists, physical chemists and physicists.

At this conference we propose to discuss the energetics of chemical reactions catalyzed by enzymes. The number of enzyme systems which have been investigated carefully from this point of view is small compared to the job still ahead. Nevertheless, enough information has accumulated regarding the chemical dynamics and energetics in biological systems to encourage the belief that the continued exploration of this field will give us a clearer picture of the chemical events in living cells.

For the biochemist, the prerequisites for the occurrence of a given chemical reaction are the same for a biological system as are those for a purely chemical system. Once the chemical structure of the participants in a reaction are known, the further study involves a determination of the factors which influence the equilibrium condition of the reaction and of the factors which affect the speed with which equilibrium is attained. The position of the equilibrium is a function of the energetics of the reaction and thermodynamic theory states that the reaction will approach equilibrium spontaneously only when the process involves a decrease in the free energy of the system. The importance of the knowledge of the sign and magnitude of the free energy change in biochemical reactions needs no emphasis at this point, since this question will figure importantly in the papers to be presented today and tomorrow.

What distinguishes biochemical reactions from chemical reactions in general is the means provided for the rapid attainment of equilibrium. Biological systems are uniquely characterized by the fact that they are equipped with highly specific catalysts, the enzymes. Although, on a thermodynamic basis, a very large number of reactions may be theoretically possible in a given biological system, the presence of enzymes will result in the selective catalysis of only a limited number of these reactions. This enzymatic catalysis of selected chemical reactions thus determines the course of metabolic processes. It is clear, therefore, that in order to elucidate the course of chemical reactions in a biological system, it is essential to recognize the enzymes that are active in that system.

Much of the progress in our knowledge regarding the chemical events in living systems has been due to the advances made in the isolation and purification of enzymes. The impetus for this development came from the successful crystallization of urease by Sumner and of several of the proteolytic enzymes by Northrop and his associates. To date, about twenty enzymes have been obtained in crystalline form; the most recent success has been the crystallization of muscle phosphorylase by Green, Cori and Cori. In addition, several enzymes which have hitherto resisted crystallization have been obtained in a high degree of purity. The contributions of Warburg are perhaps the outstanding example of the way in which the availability of purified enzymes makes possible a clearer understanding of biochemical reactions. It is becoming increasingly apparent, therefore, that future advances in our knowledge of the chemical dynamics of living matter depend, in the first place, on the isolation, purification and study of the catalytic action of individual enzymes.

One important conclusion that has emerged from the studies on purified enzymes is that the catalytic activity was found, in each case, to be associated with a protein. The idea of the protein nature of enzymes, proposed by Traube in 1860 and restated by Emil Fischer in 1907, was widely accepted by biochemists until the prestige of the Willstätter school swayed opinion the other way. In the face of the work with crystalline enzymes, we have now returned to the view that enzymes are proteins.

In one sense, this is unfortunate, since it means that the complete clarification of the chemical constitution of enzymes depends on the solution of the problem of the structure of proteins. It looks as though we shall have to await further progress in the field of protein chemistry before we shall be able to discern those structural features that distinguish the enzymes from catalytically inactive proteins.

In another respect, however, what we do know about the chemical composition of proteins gives a clue to the basis for the extreme differences in specificity between enzymes. It seems probable that the precise specificity of each enzyme is the expression of a particular chemical structure, perhaps a particular arrangement of amino acid residues in the enzyme molecule. Certainly, of the known constituents of living matter, there are few which are capable of the variations in chemical structure that are possible for proteins.

The structural elements of an enzyme protein that are responsible for its catalytic power are unknown. The assumption is made that there is an active catalytic center and that the first step in catalysis involves the combination of the substrate with the active center of the enzyme to form a dissociable enzyme-substrate complex. According to this view, during the period of its contact with the enzyme, the substrate is activated and this activated substrate molecule then undergoes rapid reaction with another substance. The reality of the enzyme-substrate combination, a concept clearly developed by Michaelis in 1913, is unquestioned today. Recent studies by Keilin, Stern, and Chance on catalase and peroxidase have provided striking additional evidence for the existence of enzyme-substrate compounds.

Mention of the enzymes catalase and peroxidase recalls the fact that each of them represents a complex protein in which a so-called prosthetic group, in this case a hemin, is linked to a specific protein. Both the catalytic activity and the specificity of action depend on the nature of this protein. The term prosthetic group has also been used to designate molecules such as the pyridine nucleotides and the alloxazine nucleotides which combine reversibly with specific proteins and, as a result, undergo rapid oxidation-reduction reactions. The brilliant researches on the chemical nature of these low molecular weight substances have perhaps tended to obscure the fact that the essential catalytic activity resides in the protein to which they become attached. The so-called prosthetic group is usually a partner in the chemical reaction catalyzed by the enzyme. The active catalytic center is represented by structural elements in the protein with which the prosthetic group combines.

Since we do not know the chemical nature of the active centers of enzymes, it is impossible today to visualize the mechanism of activation of the substrate in terms of the chemical structure of intermediate compounds, as has been possible in certain cases of catalysis by simple organic compounds. Langenbeck, for example, has found that certain simple organic substances can catalyze chemical reactions for which enzyme catalysts were known. This empirical approach is of considerable interest, but the identity, or even the similarity, of the active group in the model catalyst and in the enzyme still remains to be demonstrated.

Recent years have seen several developments in the application of the modern theories of chemical kinetics to the problems of enzyme catalysis. The view has been put forward by Haber and Willstätter and later by Moelwyn-Hughes and Waters that enzymes act as initiators of chain reactions. In addition, Eyring, Stearn and Medwedew have approached the question from the point of view of quantum chemistry. It is clear that future progress in our understanding of the mechanism of enzyme action depends greatly on the efforts of physical chemists to establish a consistent theory of chemical catalysis.

Pending these attempts to define the mechanism of enzyme action in the language of physics and chemistry, the biochemist will probably continue to explore the role of enzymes in biological systems. In particular, he will seek to discover the nature of the enzyme catalyzed chemical reactions that occur in such systems. The prominent reactions in the cell are the processes of hydrolysis and condensation and of oxidation-reduction. In addition to these major reactions, organic substances may undergo a variety of other reactions such as amination and deamination, alkylation and so forth. In the cell, many of these reactions are going on continuously and simultaneously. Large molecules such as the proteins are constantly being broken down into their constituents. These degradative processes are balanced by synthetic reactions. Since, in many cases, these synthetic reactions require energy for their occurrence, they must be coupled with other processes which will provide the driving force for synthesis. The steady state which is established represents, therefore, a dynamic equilibrium in which enzymes are continuously catalyzing not only degradation and regeneration reactions but also those chemical reactions which make available, for synthetic processes, the energy obtained from the environment. It is characteristic of the steady state in living matter that many of the individual chemical systems do not attain thermodynamic equilibrium, but are poised at concentration ratios different from those expected on the basis of thermodynamic data. Thus, the position of the steady state is determined, not only by the thermodynamic potentials, but also by the kinetics of the individual enzyme catalyzed reactions.

These conferences were inaugurated a number of years ago in order to provide an opportunity for workers in actively developing fields to meet and to discuss their problems. The active development of the subject of the present conference is unquestioned. It may not be too much to hope that this discussion concerning the energetics of biochemical catalysis will in itself serve as a catalyst for future research

ENERGY RELATIONSHIPS OF THE OXIDATIVE ENZYMES

By Eric G. Ball
Harvard Medical School, Boston, Mass.

Life, from the viewpoint of the physical chemist, may be defined as a manifestation of the transformation of energy by the living organism. The plant, for example, through the agency of chlorophyll, is able to transform solar energy into potential chemical energy by the synthesis from CO_2 and water of carbohydrates, fats, and proteins. The animal, on the other hand, is able to release this stored solar energy by converting these plant products back again into CO_2 and water. It is, of course, by this procedure, which we term metabolism, that the animal obtains the energy by which he moves himself and surrounding objects, by which he maintains a body temperature usually higher than his environment, and by which he performs a multitude of processes whose study is the delight of the physiologist and which constitute life.

During the past twenty years, our knowledge of the metabolic processes whereby carbohydrates, fats, and proteins undergo degradation in the animal body has increased greatly. To be sure, we are still far from a complete understanding of the processes involved, but we have advanced far enough so that probing fingers are already beginning to search for the mechanism whereby this energy release is geared to perform the functions essential to life. Let us, therefore, consider briefly what we know today concerning the oxidative reactions by which foodstuffs are converted into CO_2 and water and see what deductions we may draw concerning the amount of energy thereby released and its utilization by the living cell.

In the consideration of the energy release of foodstuffs during oxidation, it has been the custom to write an over-all equation for the reaction such as the one shown here for glucose:

$$C_6H_{12}O_6 + 6\,O_2 \longrightarrow 6\,CO_2 + 6\,H_2O;\ \triangle\,H = -673{,}000 \text{ calories} \quad (1)$$

In this process of combustion, we have learned that whether it takes place in the body or *in vitro*, the same amount of heat, $\triangle$ H, is evolved, and this is 673,000 calories for each gram molecular weight of glucose burned. Now, the expression of energy release as heat in this equation, though undoubtedly satisfactory for the purpose of calculating food requirements of an individual, is not suitable if we wish to discuss accurately the energy relationships of the oxidative processes in the body. Animals are not heat engines and as such can utilize only the free energy, Δ F, of such reactions to carry out their various physiological functions. It is, thus, with Δ F that we must deal in our considerations of the energy of a system which is convertible into useful work in the animal body. For an isothermal process, the two quantities Δ F and Δ H are related to one another by the fundamental thermodynamic equation

$$\Delta F = \Delta H - T\Delta S \qquad (2)$$

where T is the absolute temperature and Δ S is the entropy of the system under consideration.

There are four general methods which have been employed in determining the value of the free energy change, Δ F, in various chemical reactions. As stated by Parks and Huffman[9] these are:

1. The measurement of the equilibrium constant, K, in a "reversible" reaction and the calculation of ΔF by the relation $\Delta F = -RT\ln K$.
2. The determination of the reversible electromotive force, E, of an electrical cell involving the reaction in question, in which case $\Delta F = -NFE$.
3. The determination of heat capacities down to very low temperatures and the utilization of these results with other thermal data to calculate ΔF by means of the third law of thermodynamics.
4. The combination of suitable chemical equations with known ΔF values to give the reaction in question.

We shall have occasion to use Δ F values calculated by all four methods in the discussion that follows.

Let us now take a typical foodstuff, glucose, and calculate as follows the free energy for the reaction in which it undergoes combustion:

$$C_6H_{12}O_6\ (s) \longrightarrow 6\ C + 6\ H_2 + 3\ O_2;\ \Delta F = 215{,}800 \text{ cal.} \qquad (3)$$

$$6\ C + 6\ O_2 \longrightarrow 6\ CO_2\ (g);\ \Delta F = 6 \times -94{,}100 = -564{,}600 \text{ cal.} \qquad (4)$$

$$6\ H_2 + 3\ O_2 \longrightarrow 6\ H_2O\ (l);\ \Delta F = 6 \times -56{,}560 = -339{,}360 \text{ cal.} \qquad (5)$$

$$C_6H_{12}O_6\ (s) + 6\ O_2 \longrightarrow 6\ CO_2\ (g) + 6\ H_2O\ (l);\ \Delta F = -688{,}160 \text{ cal.} \qquad (6)$$

The values employed in equations (3), (4), and (5) are taken from Parks and Huffman.[9] The summation of these equations yields equation (6) and a value for Δ F = — 688,160 calories. There are thus released 688,160 calories capable of performing work when one gram molecule of glucose undergoes oxidation to CO_2 and H_2O. It should be noted that though Δ F is nearly equal to Δ H for this reaction (equation 1), this is not true for all reactions. For example, in the conversion of glucose to lactate, Δ F is approximately 50 per cent greater than Δ H.

Now equation (6) tells us nothing concerning the mode of oxidation of glucose in the animal body. We know, however, from the first law of thermodynamics that regardless of the pathway by which this oxidation occurs, the same number of calories will be released as free energy. Actually, however, the amount of free energy released in the combustion of glucose in the animal body will not be exactly equal to that stated in equation (6). This is because the equation as written applies only when all substances participating in it are at the standard state and the reaction occurs at 25° C. To obtain the corresponding free energy for the process as it occurs in the mammalian body, corrections should, of course, be made so that all the participants are at the concentration met with in the body and at a temperature of 38° C. Since these corrections would, however, amount to but a small fraction of the total free energy, they will be ignored for the sake of simplicity in most of the discussion that follows.

Now, before we can proceed further with our consideration of energy relationships, it is necessary for us to inquire into what is known concerning the mechanism of biological oxidations in the animal body. We may schematize the chief process by which foodstuffs appear to be oxidized in the living cell in the manner shown in FIGURE 1. The food-

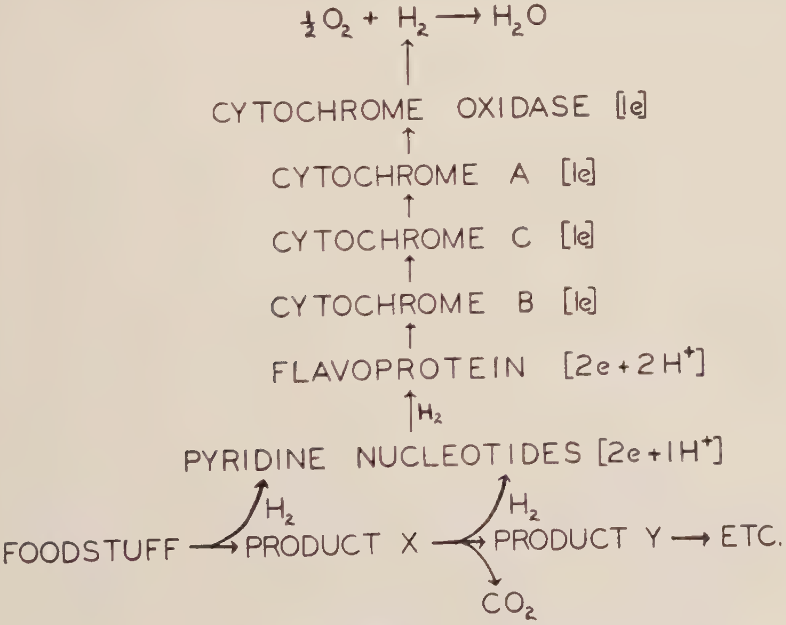


FIGURE 1.

stuff, on entering the cell, undergoes an enzymatic reaction in which two hydrogen ions and two electrons (or two hydrogen atoms) are removed and in which one of the pyridine nucleotides is reduced. There then follows a series of oxidations and reductions involving a flavoprotein, the three cytochromes, and cytochrome oxidase, by which the pyridine nucleotide is reoxidized and the hydrogen ions and electrons are transferred to oxygen with the formation of water. The dehydrogenated product (X) of the foodstuff then undergoes a similar enzymatic reaction in which a different enzyme from the first one is required, though the same pyridine nucleotide may again act as coenzyme. Two hydrogens are again removed and their transfer to oxygen then follows the same pathway as the previous two. The breakdown of the foodstuff thus continues to completion, step by step, with the liberation of two hydrogen atoms at each step. At various stages in the process of the foodstuff breakdown, the products formed may not only take on or give off a molecule of water or of phosphate, but also lose a molecule of CO_2 by a decarboxylation reaction. The end products of the reaction then are CO_2 and water. Note, however, that all of the molecular oxygen that participated in the reaction appears as water and none goes directly to form CO_2. The oxygen that finally appears in the CO_2 was either in the foodstuff to start with or is oxygen that was added as water (or phosphate) during the reactions. We shall return to this point later.

It should be noted also that though all of the oxidative enzymes participate in the passage of electrons from foodstuff to oxygen, they all do not participate in the passage of hydrogen ions. The cytochrome members of the chain transfer only electrons and, moreover, they are able to transfer only one electron at a time. The flavoproteins and pyridine nucleotides, on the other hand, transfer both hydrogen ions and electrons and are capable of transferring a maximum of two electrons at a time. The hydrogen atoms that combine with oxygen to form water must thus be obtained by combining electrons passed through the oxidative enzyme chain with hydrogen ions withdrawn from the environment.

Now during each of these many reactions, energy has been liberated so that the next question we may ask is what proportion of the total energy can be accounted for in each one of these reactions. In order to answer this question, let us focus our attention more closely on the oxidative enzymes which are participating in these reactions. Each one of these compounds is capable of undergoing a reversible oxidation and reduction and, therefore, forms a system whose oxidation-reduction potential should be measureable in relation to any other system. Unfortunately, the direct measurement of the oxidation-reduction potential of most of these systems is fraught with technical difficulties that need not, however, be detailed here. Let it suffice to say that it has been possible to obtain at least approximate values for all of them except cytochrome oxidase. In TABLE 1, the oxidation-reduction potentials of these systems along with those of the oxygen and hydrogen electrodes at pH 7.0 are listed. It will be noted that the potentials of these systems decrease progressively in the order listed, and it is this relationship which formed the basis for their arrangement in FIGURE 1. In this connection, it is highly important to mention the fact that these systems show a marked specificity of interaction where this can be tested. For example, the reduced pyridine nucleotides will not react directly with oxygen nor with cytochrome c, but only with flavoprotein. Though the evidence is not complete, we shall assume each compound listed here will react only with the compound listed immediately above or below it. We may now calculate the potential differences between neighboring pairs of these systems and obtain the free energy released on their interaction by means of the equation

$$\triangle F = -nFE$$

where n equals the number of electrons exchanged, F is the Faraday, and E is the potential difference expressed in volts. Taking the Faraday as equal to 23,068 calories, we obtain the free energy values given in the last column of TABLE 1.

Inspection of this table indicates that the number of calories released varies greatly, depending upon the pair of systems which are interacting. For example, the interaction of cytochrome a and cytochrome c, according to these calculations, yields only 460 calories while 9,200 calories are released when two electrons are transferred from the pyridine nucleotides to flavoprotein. Are we now to conclude that these values represent the maximum energy to be obtained upon the interaction of these systems in the animal body? The answer is in the negative, of course, because our calculations are based upon the E'_0 values of these systems and thus represent only the amount of energy released upon the interaction of these systems when they are all 50 per cent oxidized (or reduced). It is doubtful if this is the relationship between them during the metabolic activity of the cell. It is possible, therefore, that more, or even less, energy is released in the living cell by the interaction of any pair of these systems than is calculated here. Unfortunately, we have no information which will permit us to say pre-

TABLE 1

E'_0 AND ΔF VALUES FOR THE INTERACTION OF THE OXIDATIVE ENZYMES AT pH 7.0 AND 30° C.

	E'_0	ΔF per electron	
O_2	+0.81 v	0.52 v	−12,000
Cytochrome oxidase	?		
Cytochrome a	+0.29 v	0.02 v	−460
Cytochrome c	+0.27 v	0.31 v	−7,150
Cytochrome b	−0.04 v	0.04 v	−920
Flavoprotein	−0.08 v	0.20 v	−4,600 (× 2 = −9,200)
Pyridine nucleotide	−0.28 v		
H_2	−0.42 v		−25,130 cal.

$\Delta F = -nFE = -1 \times 23{,}068 \times$ voltage difference

The source of the E'_0 values of all systems except the pyridine nucleotide is given by Ball.[1] The value for this system is that calculated by Borsook.[5] The E'_0 value for this system of −0.26 v reported by Ball and Ramsdell[2] is undoubtedly too high, since subsequent tests have indicated that the sample of pyridine nucleotide employed by them was not 100 per cent pure.

cisely what amount of energy is released by the interaction of any pair of these systems in the living cell. At best, the calculations given here are useful only to indicate the rough magnitude of possible energy release in the interaction of these various systems in the animal body. For example, if we accept 12,000 calories as the energy necessary to form a high energy phosphate bond, it would seem reasonable to suppose that the reactions most likely to furnish this amount of energy would be those between the pyridine nucleotides and flavoprotein (two electrons), cytochromes b and c, or of cytochrome oxidase with either O_2 or cytochrome a, depending on the potential of the cytochrome oxidase system. In any one cycle, of course, all three of these pairs could not each supply 12,000 calories, since, to obtain 12,000 calories from any one of these pairs, would mean a corresponding decrease in energy release elsewhere in the chain, on the basis of the calculations in TABLE 1. By summation of the energy release for all of these systems, we see that only some 25,000 calories are capable of being released for each electron transferred through the total chain so that, no matter how we choose to subdivide it, this is the total amount of energy available per cycle. In view of this fact and the number of participating systems in the chain, it seems not unreasonable to assume that the 12,000 calories required to form a high energy phosphate bond represent about the maximum individual parcel of energy to be drawn off from oxidative reactions. However, it must not be forgotten that cytochrome oxidase, the three cytochromes, and the flavoprotein reacting in this chain are all apparently intimately associated in a submicroscopic particle within the living cell. It is possible, therefore, that energy release should not be discussed in terms of pairs of these systems, since it is conceivable that a close association of these enzymes permits energy exchanges to occur that might not otherwise be possible. We shall be on more substantial ground if we deal with the total amount of energy released by interaction of all of the oxidative enzymes in the chain during the passage of one electron from the half reduced pyridine nucleotide system to oxygen.

Let us then return to the oxidation of glucose and attempt to calculate what percentage of the total free energy of this reaction will be released by the interaction of the oxidative enzymes. We have seen that, in any biological oxidation, all the molecular oxygen that enters the reaction will appear as H_2O. Therefore, in the oxidation of glucose in the living cell, we will write the equation

$$C_6H_{12}O_6 + 6\,O_2 + 6\,H_2O \longrightarrow 6\,CO_2 + 12\,H_2O;\ \triangle F = -688{,}160 \text{ cal.} \quad (6a)$$

so that the six oxygen molecules that enter the reaction will produce 12 H_2O. If this equation is to balance, however, we must write 6 H_2O on the left-hand side and we may, therefore, say that at least 6 water molecules enter into the intermediate reactions which take place when glucose is oxidized in the animal body. An alternative method would be to write the equation as involving the participation of phosphoric acid with the production of high energy phosphate bonds as in equation (7).

$$C_6H_{12}O_6 + 6\,O_2 + 6\,H_3PO_4 + 6\,H^+ \longrightarrow 6\,CO_2 + 12\,H_2O + 6 \sim H_2PO_3 \quad (7)$$

Parenthetically, it must be pointed out that this equation must not be interpreted as defining the limits for the number of high energy PO_4 bonds formed per mole of glucose burned. Regardless, however, of the manner in which we write this equation, the main fact that concerns us here is that the conversion of 6 molecules of oxygen into water requires the participation of 24 electrons and 24 hydrogen ions.

$$6\,O_2 + 24\,e + 24\,H^+ \longrightarrow 12\,H_2O \quad (8)$$

This means that 24 electrons must be transferred from the substrate through the oxidative enzyme chain to O_2 for each mole of glucose burned in the body. Now, if we assume that all the energy released is by electron transfer, we may calculate that the maximum free energy that could be released per electron transferred would be the total free energy of 688,160 calories divided by 24, or 28,670 calories. To be sure, all energy release is probably not by electron transfer, but since we have presented in TABLE 1 calculations showing the amount of free energy released by the exchange of one electron between the various oxidative enzymes, we are now able to obtain some idea of the proportion of the total energy that may be so released. For example, the transfer of one electron from the pyridine nucleotides to O_2 was calculated to release 25,130 calories. A simple calculation shows this to be 87.6 per cent of the total maximum free energy theoretically available for release. It must be pointed out, however, that this value represents nearly the maximum percentage of the total free energy that could be released by the passage of electrons through the chain of oxidative systems. Actually, somewhat less than this amount may be so released, because we know that the potential of the pyridine nucleotides lies below some of the reversible substrate systems with which they react.

In all fairness, then, let us take the next enzyme system in the chain, flavoprotein, and repeat the calculations. The transfer of one electron from flavoprotein to O_2 can be calculated to release 20,530 calories and this represents 71.6 per cent of the total free energy available. It seems reasonable to assume that this represents the minimum possible percentage of the total free energy released through the oxidative enzymes since, at the E'_0 value of the flavoprotein system, the ratio of oxidized pyridine nucleotide to the reduced form would be about five million to one. Thus, we reach the conclusion that somewhere between 72 and 88 per cent of the total free energy released during the oxidation of glucose in the animal body can be accounted for by interaction of the oxidative enzymes. The remainder of the energy, amounting to 12–28 per cent of the total, can then be said to be released during the interaction of the substrates with the pyridine nucleotides, or in reactions in which the removal of electrons and hydrogen ions is not involved. A good example, of course, of the latter type of reaction is the anaerobic conversion of glucose into 2 lactate molecules. Here, there is merely a molecular rearrangement without the loss of any electrons, and it can be calculated that under body conditions some 50,000 calories per mole of glucose are liberated by the reaction. To be sure, diphosphopyridine nucleotide participates in this reaction, but it acts merely as a shuttle to redistribute electrons and hydrogen ions. Thus, the conversion of glucose into lactate yields only some 7 per cent of the total free energy to be obtained by complete glucose combustion. It is interesting to note that, even after such an anaerobic energy release, the product of the reaction, lactate, still is oxidized by the same pathway involving a pyridine nucleotide. Now, if all products of anaerobic reactions must still lose their electrons by this pathway, then we may conclude that the maximum anaerobic free energy release for glucose under any circumstances cannot be greater than 12-28 per cent of the total free energy released on oxidation.

Similar calculations may also be made for the combustion of a fatty acid. Using palmitic acid as an example, the equations given here summarize the pertinent data.

$$C_{16}H_{32}O_2 + 23\ O_2 + 30\ H_2O \longrightarrow 16\ CO_2 + 46\ H_2O; \quad \triangle F = -2{,}330{,}560 \text{ cal.} \qquad (12)$$

$$23\ O_2 + 92\ e + 92\ H^+ \longrightarrow 46\ H_2O \qquad (13)$$

$$-2{,}330{,}560 \text{ cal.} \div 92\ e = -25{,}332 \text{ cal. per electron transferred} \qquad (14)$$

One electron from pyridine nucleotides to $O_2 = -25{,}130$ cal.

$$\frac{25{,}130 \text{ cal.}}{25{,}332 \text{ cal.}} = 99.2 \text{ per cent of total energy} \qquad (15)$$

One electron from flavoprotein to $O_2 = 20{,}530$ cal.

$$\frac{20{,}530 \text{ cal.}}{25{,}332 \text{ cal.}} = 81.0 \text{ per cent of total energy} \qquad (16)$$

If the oxidation of palmitic acid follows the pathway we have outlined in FIGURE 1, then we may say that some thirty molecules of water must enter the reaction and 92 electrons will be transferred through the oxidative chain for each mole of acid oxidized. A total of 2,330,560 calories will be released as free energy, or a maximum of 25,332 calories for each electron transferred. By means of calculations similar to those used for glucose, we reach the conclusion that somewhere between 81 and 99 per cent of the total free energy would be released by means of the interaction of the oxidative enzymes when 1 mole of palmitic acid undergoes oxidation in the animal body. This leaves only 1 to 19 per cent of the total free energy to be released by other mechanisms, which is considerably less than was found in the case of glucose. Since we have postulated that it is from this portion of the total free energy that anaerobic processes should spring, it is interesting to speculate that perhaps this narrower margin in the case of fatty acids accounts for their apparent inability to furnish energy by anaerobic reactions.

I should perhaps pause at this point to acknowledge the fact that we are not by any means certain that the oxidation of all foodstuffs follows the outlined pathway. This is especially true of the fats, where our knowledge of the actual mechanism involved in their oxidation is indeed paltry. We do know, however, that some 90 per cent of the overall respiration of the cell may be blocked by cyanide, and this suggests that the cytochrome-flavoprotein chain is indeed responsible for the bulk of all oxidative processes. The 10 per cent cyanide insensitive respiration would appear to be due largely to flavoprotein systems, such as d-amino acid oxidase and xanthine oxidase, which are capable of direct reaction with O_2. Whether any of the energy released by the action of such flavoprotein systems is utilizable by the organism remains to be answered. However, the potential of such flavoprotein systems is probably not greatly different from that of the flavoprotein participating in the cytochrome chain, so that the bulk of the energy release still occurs in the transfer of electrons and hydrogen ions between a flavoprotein and oxygen. It may well be, of course, that oxidative processes still unknown to us, and whose pattern is markedly different from the dehydrogenation type so familiar to us today, do occur in the body. If so, I can only hope that the deductions made here will so stimulate you to prove me wrong that the discovery of such mechanisms will be greatly hastened.

The best conclusion that we can, therefore, reach today is that the energy set free during the oxidation of foodstuffs is probably released in definite-sized parcels, a step at a time, with the bulk of the energy being released by the interaction of the oxidative systems. Whether the cell can further subdivide these parcels of energy for its use, we do not know. One interpretation of the all-or-none law of muscle and nerve action might be that these cells can respond only by expending a full parcel of energy whenever their threshold of stimulation is reached. Even so, the minimum size of this energy parcel is unknown. We also have little or no information to tell us whether the energy released at each one of these steps is capable of being harnessed for work by the cell. In some steps, for all we know, the energy may appear as heat to be wasted or utilized to maintain body temperature. Most of the information that we possess concerning energy utilization revolves around the high energy phosphate bonds formed in glycolytic reactions, and this topic lies outside my province. We may, however, garner some clues as to whether the energy released by the interaction of the oxidative enzymes is utilized by an inquiry into the efficiency of the living cell in performing its various functions. For this purpose, let us examine the relative efficiencies of certain different physiological processes.

The process of secretion or excretion is one in which certain calculations concerning efficiencies of energy utilizations may be made. Borsook and Winegarden[6] have calculated that the formation of one liter of urine requires about 700 calories. These same authors[6] have calculated the efficiency of the kidney by assuming that a liter of urine is formed in 24 hours and that the total oxygen consumption of the kidney in this period of time furnishes energy which is all expended in the process of excretion. They reach the conclusion that the kidney performs its work with an efficiency probably not greater than 1–2 per cent. I have made similar calculations for the formation of pancreatic juice based solely upon the difference in its ionic composition and that of plasma and have obtained a value of 133 calories required for each liter of juice formed. Using the data of Still, Bennett, and Scott[10] for the difference in the O_2 consumption of the resting and secreting gland, it is possible, assuming an R.Q. of 1.0, to calculate that, in the formation of 1 liter of pancreatic juice, approximately 3800 calories are consumed. The efficiency of secretion of pancreatic juice is thus of the order of 3–4 per cent. A similar calculation may be made for the gastric juice, and shows that about 1500 calories are required for each liter of juice formed. Data on the oxygen consumption of the gland are apparently not available, but if we assume that Davenport's[7] explanation of the formation of the juice is correct, then one mole of CO_2 is produced for each mole of H^+ concentrated. With an R.Q. of 1.0, we may calculate that the amount of oxygen consumed in the process would be equivalent to 17,000 calories, and hence the efficiency of the process is of the order of 9 per cent. We thus reach the conclusion that the over-all efficiency of processes of excretion or secretion lies within the range 2–10 per cent. It is thus possible for us to postulate that, in such processes, either (1) that all the various steps in the oxidative process contribute inefficiently to the supply of the energy requirements, or (2) that the energy of only certain reactions can be used efficiently and that the rest is wasted. Whether the interaction of the oxidative enzymes can furnish energy for such processes is thus, at the present time, debatable.

We may next consider a process in which energy is converted into mechanical work. Benedict and Cathcart[4] have presented data to show that, in man, muscular work may be performed with an efficiency as high as 20 per cent. Now, we have seen that, on the average, only some 20 per cent of the total energy released by the oxidation of foodstuffs can be attributed to reactions not involving the oxidative enzymes. We must, therefore, conclude that either such processes are 100 per cent efficient, or that some of the energy released by the interaction of the oxidative enzymes is utilizable. A consideration of the over-all efficiency of oxidative phosphorylation processes makes the latter explanation seem most reasonable.

Belitzer and Tsibakowa[3] and more recently Ochoa[8] have reported that during the oxidation of carbohydrate by heart muscle, as many as six energy-rich phosphate bonds may be formed for each molecule of oxygen consumed. Accepting 12,000 calories as the energy needed to form one energy-rich phosphate bond, we may calculate that, in the combustion of one mole of glucose where 6 moles of oxygen are utilized, some 36 such bonds representing 432,000 calories will be formed. This corresponds to some 63 per cent of the total free energy released upon the oxidation of one mole of glucose. Hence, the conclusion seems inescapable that some of the energy released by the interaction of the oxidative enzymes is being utilized to form high energy phosphate bonds. Thus, the 20 per cent over-all efficiency of muscular work must be attributed to energy wastage during the muscle's utilization of phosphate bond energy as well as in its inefficient use of energy from oxidative processes.

In summary, then, it would appear from our present-day knowledge that the bulk of the energy liberated in the oxidation of foodstuffs is released by the interaction of the oxidative enzymes and that some of the energy so released may be utilized by the living organism. However, many puzzling questions still remain to be answered. There is lacking any knowledge of the factors which control the release of energy by such processes to meet the fluctuating needs of the cell. We are also in the dark concerning the mechanisms by which the energy released is geared to useful purposes within the cell. The vitamins have already been brought into the picture, perhaps the hormones are next. Surely, exciting discoveries lie ahead for those who are delving into these problems, and we may await future developments in this field with great expectancy. May the day soon come when our undivided efforts can again be directed toward the solution of such problems.

REFERENCES

1. **Ball, E. G.**
   1939. Symposia on Quantitative Biology **7**: 100.
2. **Ball, E. G., & P. A. Ramsdell**
   1939. Jour. Biol. Chem. **131**: 767.
3. **Belitzer, V. A., & E. T. Tsibakowa**
   1939. Biokhimiya **4**: 516.
4. **Benedict, F. G., & E. P. Cathcart**
   1913. Muscular Work. Carnegie Inst. of Wash., Publ. No. **187**.
5. **Borsook, H.**
   1940. Jour. Biol. Chem. **133**: 629.
6. **Borsook, H., & H. M. Winegarden**
   1931. Proc. Nat. Acad. Sci. **17**: 3, 13.
7. **Davenport, H. W.**
   1943. Gastroenterology **1**: 383.
8. **Ochoa, S.**
   1943. Jour. Biol. Chem. **151**: 493.
9. **Parks, G. S., & H. M. Huffman**
   1932. The free energies of some organic compounds. New York.
10. **Still, E. V., A. L. Bennett, & V. B. Scott**
    1933. Am. Jour. Physiol. **106**: 509.

ENERGY RELATIONSHIPS IN GLYCOLYSIS AND PHOSPHORYLATION

By Otto Meyerhof
*University of Pennsylvania, School of Medicine,
Department of Physiological Chemistry,
Philadelphia, Pa.*

Our knowledge of the energy relationships in glycolysis and phosphorylation developed in close connection with the problem of the energy-transferring chemical reactions of muscle activity. I may be allowed, therefore, to start with that topic. Leaving aside the concepts of the so-called classical period of physiology and starting with the myothermic work of Hill and Hartree about 1920, we may broadly distinguish three historic periods in these investigations.

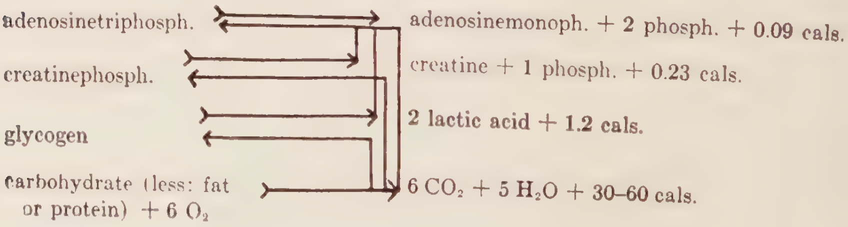
In the first period, which fills a little more than a decade from 1920 to 1932, the relationship of the anaerobic muscle metabolism to the oxidative metabolism, as well as to the production of heat and work, was studied in the living isolated muscle. As a result, the three most important reversible systems connected with muscular activity were brought to light: (1) Splitting of carbohydrate to lactic acid and aerobic resynthesis; (2) splitting of creatinephosphate into creatine and phosphate and anaerobic and aerobic resynthesis; (3) splitting of adenylpyrophosphate and anaerobic and aerobic resynthesis.

In the second period, by a close study of the enzymatic breakdown of carbohydrate in solution, the intermediates in this breakdown were isolated, transfer of phosphate and hydrogen could be ascribed to single reaction steps, and the equilibria in solution could be established, as well as the total energy of the different steps. This period again roughly fills a decade and closes with the latest discovered intermediate, 1,3 diphosphoglyceric acid, by Warburg, Christian and Negelein in 1939.

The final period, in which we now stand, thus far has shown three different trends: firstly, to develop a more general theoretical view of the energy transfer by means of energy-rich phosphate bonds, especially in the contributions of Lipmann[1] and Kalckar[2] to this subject; secondly, to generalize the relation between hydrogen transfer and phosphorylation to all oxidative steps, that is, to the respiration of carbohydrate as well as to the oxidoreduction. In addition to the work of the above investigators, one may mention several papers from Cori's laboratory[3] and also some by Ochoa.[4] The third tendency has been to apply our knowledge to a final understanding of the contraction mechanism of muscle by means of the hypothesis that the phosphate-bond energy is finally transferred to the contractile protein itself. I here refer to the work of Engelhardt, followed by that of J. Needham and others. Although these attempts, at the moment, are highly speculative, eventually they may lead to the clue to this last energy transfer. I shall restrict my report mainly to the first two periods, in which I was able to participate more actively. I expect that Dr. Kalckar, as a representative of a younger generation, will devote more of his time to the more recent developments.

Before going into details, I may state that the heat of the different reactions can be measured by direct calorimetry with considerable accuracy,—much more accurately than by calculation from measured combustion heats, which involve small differences between large values. The latter method is subject to great errors, especially if the heats of solution and dilution are not known or if the effect of phosphorylation is disregarded. But in some cases, we must rely on the latter procedure, where, for any reason, direct calorimetry is inapplicable. All energy-rich phosphate bonds were found by direct calorimetry. On the other hand, the change of free energy in the reaction can be measured directly only in those instances where the equilibrium is not shifted too far to one side (with the proportion of the reactants not higher than 99:1), so that the equilibrium concentrations can still be accurately measured. This corresponds, for monomolecular reactions, to K values between 10^2 and 10^{-2}; for bimolecular splitting or synthesis, to K values between 10^4 and 10^{-4}. For our ranges of temperature and concentration, $\triangle F$ is then always smaller than approximately 5000 calories. Since phosphorylation potentials cannot be determined by means of electromotive force measurements like oxidation potentials, free energy changes of those types of phosphate transfer which yield great energy ($-\triangle F$ over 5000 calories), and which are more interesting, must be calculated more or less with the help of thermal data.

I shall now come to the first historic period of which I have spoken. How are we to picture, with our present-day knowledge, the over-all energy exchange of a working muscle under aerobic and anaerobic conditions? Here the general scheme which summarizes the results of this period still holds.[5]


adenosinetriphosph.
creatinephosph.
glycogen
carbohydrate (less: fat or protein) + 6 O₂
adenosinemonoph. + 2 phosph. + 0.09 cals.
creatine + 1 phosph. + 0.23 cals.
2 lactic acid + 1.2 cals.
6 CO₂ + 5 H₂O + 30–60 cals.

The calories here refer to the available energy stores for the different reactions in one gram of unfatigued frog muscle. Every reaction below that shown in the uppermost line of the diagram is farther away from the immediate supply of energy for work, and has, at the same time, a larger store of energy. Moreover, it is coupled with the reversal of the adjacent reaction, that is, with the recombination of the split products. But it is also coupled with all others by shunts. This is very important. For instance, the oxidation of carbohydrate can bring about the synthesis of creatine-phosphate not only by way of intermediate lactic-acid formation, but directly as well. Under strict aerobic conditions, this applies not only to the alactacid muscle poisoned with iodoacetic acid[5a] but to the normal muscle as well. Such a muscle, apparently, does not form lactic acid at all, and the oxidation serves for immediate resynthesis of creatinephosphate. Lactic acid formation is already an emergency mechanism, which, however, is set in motion under normal conditions in a muscle working *in situ;* especially, until the blood supply is adapted to the sudden increase of the demand of oxygen by the start of work. Intermediate lactic acid formation, therefore, is in response to the lack of oxygen, while breakdown of creatinephosphate in the working muscle occurs alike in presence and absence of oxygen.

This view is somewhat different from the older assumption that, even under normal oxygen tension, lactic acid appears as an intermediate in the active non-poisoned muscle; and it removes a curious difficulty or paradox encountered in the relationship of phosphocreatine breakdown and lactic acid formation during anaerobic fatigue of an isolated muscle.[6] In the first contractions of such a series of twitches or tetani, the quotient of $\frac{\text{creatinephosphate split (mole)}}{\text{lactic acid formed (mole)}}$ is high,—about 3 to 4. For high degrees of fatigue it is very low, probably less than 0.2. But "creatinephosphate split" in the numerator refers to that residual amount of split creatinephosphate which is found by analysis after the contraction, and which is not immediately resynthesized during lactic acid formation. From this, it must be concluded that, at first, extremely small amounts of creatinephosphate are resynthesized by coupling with lactic acid formation and, in high degrees of fatigue, nearly all newly split creatinephosphate is synthesized in that way. This paradoxical result, that anaerobic resynthesis of creatinephosphate becomes more and more evident with increasing anaerobic fatigue, and is nearly absent in the beginning, is easy to understand when one considers that the mechanism of coupling with lactic acid is only an emergency device. Usually creatinephosphate is synthesized by coupling with *oxidation.* But when a larger part is broken down and oxygen is not available, lactic acid formation sets in, which counteracts the depletion of the energy store of creatinephosphate. Theoretically, it would seem possible that muscle contraction could proceed in oxygen solely with the breakdown of adenosinetriphosphate and the subsequent resynthesis, without intermediate breakdown of creatinephosphate. The available store of .09 calories would be enough for about 30–50 single maximal contractions. But by registration with glass electrodes from the muscle surface, Dubuisson[7a] has obtained a curve of pH change, which shows an alkalinization even in the very first contractions, and this alkalinization is interpreted as a breakdown of phosphocreatine.

From these newer concepts of muscle metabolism, we can expect an approximately constant caloric quotient of lactic acid in muscle, $\frac{\text{calories produced anaerobically}}{\text{gms. lactic acid formed}}$, only with similar degrees of anaerobic fatigue. Although a quite short activity of muscle is unsuited for chemical analysis, relatively short periods of stimulation formerly gave caloric quotients of about 400 calories, while, in the highest degrees of fatigue, the quotients found were 280 to 250 calories. Since 205 calories are developed enzymatically by splitting 0.9 grams of dissolved glycogen into lactic acid [180 calories (difference of combusion heat) + 25 calories (heat of neutralisation with bicarbonate and phosphate)], the minimum of 250 calories corresponds to nearly exclusive lactic acid formation with an approximate balance of split and resynthesized creatinephosphate, and with some additional heat derived from the great neutralization heat of protein.* The higher caloric quotients correspond to additional breakdown of creatinephosphate. When the molecular heat for the enzymatic hydrolysis of creatinephosphate (11,000 calories per mole) is taken as the basis, and the muscle is analyzed for both lactic acid and creatinephosphate, it seems that the total heat is still somewhat greater than explained by this breakdown, even if the pH change and the large neutralization heat of protein is taken into account.[8a]

Finally, we may ask how the myothermic measurements of Hartree and Hill can be explained for single twitches of a muscle in oxygen when only creatinephosphate is split, as in the case of muscle poisoned by iodoacetic acid. According to Hartree and Hill, about 50 per cent of the total heat of contraction is given up anaerobically in the initial phase during contraction, while 50 per cent is delayed heat in oxygen. For each mole of oxygen, burning carbohydrate, 113,000 calories are developed; and for each mole of creatinephosphate hydrolyzed, 11,000 calories are liberated. Therefore, 5 moles of creatinephosphate must break down and be resynthesized by one mole of oxygen. This would give 55,000 calories for the initial heat, and 113,000 — 55,000 = 58,000 calories for the oxidative heat. Actually, Nachmansohn and I, in 1930,[9] found between 3.6 and 5.4 moles of creatinephosphate (averaging 4.7 moles) resynthesized for each mole of oxygen consumed, during the first part of the recovery period after short stimulation, when about 5 times as much creatinephosphate synthesized is about 5 times the lactic acid which disappears. In the minced muscle, Belitzer and Tzibakowa[10] found, that when three- or four-carbon acids were oxidized, 4 moles of creatinephosphate were resynthesized for each mole of oxygen consumed. Theoretically, on the basis of known mechanisms of phosphate transfer, the relation of 4 moles of phosphate to one mole of oxygen can be understood, while, at the present time, higher proportions cannot be interpreted by means of known coupled reactions. But, nevertheless, they seem to occur, since recently Ochoa[4] has reported the transfer of phosphate to sugar in heart extracts in the proportion of 6 moles of phosphate to each mole of oxygen. From a thermodynamic viewpoint, such high yields of esterified phosphate per mole of oxygen are possible, even if the phosphate should be stored in an energy-rich linkage as in creatinephosphate, and would not form the usual phosphoric acid ester. Oxidation during the recovery period in the isolated amphibian muscle has no higher efficiency than 50 per cent, equivalent to the creation of 4 or 5 energy-rich phosphate bonds per mole of oxygen. But, since we know that anaerobic glycolysis has an efficiency of 100 per cent in creating such phosphate bonds, special means may exist in respiration, to bring this about, such as, for instance, the large gap of oxidation reduction potential between the cytochromes and the pyridine nucleotide.

---

* The heat of neutralization of protein amounts to 12,500 calories per equivalent, or 140 calories per gm. of lactic acid, but probably very little is neutralized in this way.

The second part of my report is concerned with the results of the second historic period of our problem. In this period, the whole pathway of breakdown leading from glycogen to lactic acid was cleared up by studying the intermediate reactions in muscle extract, yeast extract, in other cell extracts and more or less isolated enzymatic systems. Two general reaction types became known. The first includes those reactions easily reversible in the absence of other additional chemical systems, which therefore display a small change of free energy. These can be called, in a special sense, "equilibria reactions." The second type includes those, which can be reversed only by coupling with a second system, where every single reaction shows a relatively great change of free energy. But, by the combination of inducing and induced reaction, a reversible system can be built up, which is nearly "ergo-neutral." In general, the first type of reaction goes on in dialyzed extracts, where the coenzyme systems are removed, while the latter type needs coenzymes, which form a part of the coupled systems. In the stationary state of lactic acid formation from glycogen, about twelve consecutive steps are passed through, of which eight belong to the first-named group, while the rest are so combined that the change of free energy therein is equal to the change of free energy in the total chain of reactions from glycogen to lactic acid. By coupling with the adenylic system as the phosphorylating coenzyme, this free energy is transferred to two energy-rich phosphate bonds per mole of lactic acid produced.

I shall not describe here the single reactions, because I have done this previously[11] and they are well-known. However, some of the equilibrium reactions, undoubtedly, are very interesting from chemical and thermodynamic viewpoints. I mention, for instance, the zymohexase reaction between hexosediphosphate and triosephosphate, which has a strong negative heat in the direction of splitting and obeys closely the Van't Hoff law of isochores. I shall restrict myself to the reactions of the second type, with appreciable yield of free energy, and shall only discuss those points where either some progress has been made in recent times or the situation is still somewhat obscure. The concept of the energy-rich phosphate bond, developed mainly by Lipmann,[1] has undoubtedly helped to clarify and systematize our knowledge in this field. At present, it will be generally agreed by all experts in this matter, that the significance of the phosphorylation of the carbohydrate intermediates is based on its thermodynamic implications. In this way, the energy of the oxidative step can be easily collected in energy-rich phosphate bonds, which, at first, form a part of the intermediates themselves. By transphosphorylations, these bonds with their high energy are transferred to the adenylic system and are stored as the labile phosphate groups in the adenosinepolyphosphoric acids or are transferred still further to creatine and are stored as creatinephosphate. They can also be transferred to other systems with or without loss of their high bond-energy or possibly they can be released, when attached to protein in doing mechanical work.

By direct measurement, we know only the heat change connected with the release of such an energy-rich bond. The molar heat for the splitting of one labile group of adenosinetriphosphate is about 12,000 calories; for creatinephosphate, it is 11,000 calories; for phosphopyruvic acid, it is 8000 calories; and for argininephosphate, also 8000 calories. Lipmann has found an indirect method for calculating the free energy change of the dephosphorylation of phosphopyruvic acid and has obtained a $\triangle F^\circ$ of $-11{,}000$ calories. Assuming that the free energy is nearly the same in all energy-rich bonds, because of their mutual equilibria, this would be the true value for the other compounds too. Since a part of the calculations of Dr. Lipmann is based on somewhat doubtful numerical values, it may be of interest to submit a new computation of this $\triangle F^\circ$ value of the phosphate bond of adenosinetriphosphate itself, from equilibria measurements made in my former Institute in Heidelberg in 1938. The equilibria measurements were published, but, so far, neither I, nor anybody else, has attempted to use them for this calculation. All figures (except one for the combustion heat of glyceric acid) are completely independent of Dr. Lipmann's numerical data. Indeed, the thermal values used by me contain uncertainties similar to those in Dr. Lipmann's computation. Nevertheless, since this calculation is based on quite different reactions and leads to about the same result, it may add some more weight to the soundness of our assumptions.

The coupling reaction, which was described in 1938,[12] shows a stoichiometric balance, in which one mole of glyceraldehydephosphate is oxidized by one mole of cozymase to 3-phosphoglyceric acid, while one mole of phosphate combines with one mole of adenosinediphosphate to form adenosine triphosphate.

3-glyceraldehydephosphate + cozymase (DP$\overset{+}{\text{N}}$) + adenosinediphosphate + H_3PO_4

⇅

3-phosphoglyceric acid + dihydrocozymase (DPNH) + adenosinetriphosphate

The equilibrium of this reason was studied in a large series of experiments with different concentrations of the reactants. Although it was demonstrated by Warburg and Christian[13] and also by Negelein and Brömel[14] that this reaction is really composed of several steps leading over 1, 3-diphosphoglyceric acid, as a connecting link between oxidation and phosphorylation, this does not matter for the calculation of the change of free energy, which is determined solely by the concentration of the initial and final products in the over-all equilibrium.

I have reproduced here the three most exact experiments, with their equilibrium concentrations. They were made under identical conditions of volume, temperature, etc., and with the same pure preparations of the different reactants. Experiment I, where all participants were used in about equivalent amounts, was made from both sides of the equation and reached nearly the same equilibrium point in both ways. It may, therefore, be taken as the most reliable. Experiment II was made only from the left side of the equation with the same initial concentrations, but with 10 millimole excess of 3-phosphoglyceric acid (PGA). Experiment III was made from the right side with 10 millimole excess of inorganic phosphate. Dihydrocozymse was determined spectrographically. In experiment I, the other components were all determined separately; in experiments II and III, they were partly derived from the value of dihydrocozymase. Glyceraldehydephosphate (GAP) is calculated from the zymohexase equilibrium.[15] The agreement of the K value is as good as can be expected.

Equilibrium of the “Coupling Reaction”*

pH, 7.8; temperature, 20° C.

(I) $K_c =$

$$\frac{1.27 \cdot 10^{-3}\ \mathrm{DPNH} \times 1.05 \cdot 10^{-3}\ \mathrm{PGA} \times 1.16 \cdot 10^{-3}\ \mathrm{ATP}}{2.13 \cdot 10^{-3}\ \mathrm{DP\overset{+}{N}} \times 0.04 \cdot 10^{-3}\ \mathrm{GAP} \times 2.6 \cdot 10^{-3}\ \mathrm{H_3PO_4} \times 2.45 \cdot 10^{-3}\ \mathrm{ADP}}$$
$= 2.8 \cdot 10^{3}$

(II) $K_c =$

$$\frac{0.62 \cdot 10^{-3}\ \mathrm{DPNH} \times 10.7 \cdot 10^{-3}\ \mathrm{PGA} \times 0.60 \cdot 10^{-3}\ \mathrm{ATP}}{2.79 \cdot 10^{-3}\ \mathrm{DP\overset{+}{N}} \times 0.045 \cdot 10^{-3}\ \mathrm{GAP} \times 3.2 \cdot 10^{-3}\ \mathrm{H_3PO_4} \times 3.0 \cdot 10^{-3}\ \mathrm{ADP}}$$
$= 3.2 \cdot 10^{-3}$

(III) $K_c =$

$$\frac{1.74 \cdot 10^{-3}\ \mathrm{DPNH} \times 1.74 \cdot 10^{-3}\ \mathrm{PGA} \times 1.74 \cdot 10^{-3}\ \mathrm{ATP}}{1.92 \cdot 10^{-3}\ \mathrm{DP\overset{+}{N}} \times 0.035 \cdot 10^{-3}\ \mathrm{GAP} \times 11.9 \cdot 10^{-3}\ \mathrm{H_3PO_4} \times 1.9 \cdot 10^{-3}\ \mathrm{ADP}}$$
$= 3.5 \cdot 10^{-3}$

For $K = 3 \cdot 10^{3}$, $\triangle F'^{\circ}_{c} = -\mathrm{RTlnK} = -1342 \times \log. K = -4700$ calories. In calculating from this equilibrium the $\triangle F^{\circ}$ for ATP →

---

* The data are taken from, (12) table V, pages 125 and 126. For experiment I, the intermediate values for the equilibrium concentrations attained from both sides are used.

ADP + H_3PO_4, I had the kind help of Dr. H. Kalckar, who especially assisted me to transform the thermal data of the reaction (phospho) glyceraldehyde + $H_2O \rightarrow$ (phospho) glyceric acid $+2H^+$ into values of free energy.

The total equilibrium of the coupling reaction may be divided into the three partial equilibria K_1, K_2, K_3, and their corresponding $\Delta F'^{\circ}$ values $\Delta F'^{\circ}_1$, $\Delta F'^{\circ}_2$, $\Delta F'^{\circ}_3$, respectively.

$$K_1 = \frac{\text{DPNH}}{\overset{+}{\text{DPN}} \times 2H^+} \qquad K_2 = \frac{\text{3-Phosphoglycerate} \times 2H^+}{\text{3-Glyceraldehydephosphate } (+ H_2O)}$$

$$K_3 = \frac{\text{ATP}}{\text{ADP} \times H_3PO_4} \qquad \text{and} \quad \Delta F'^{\circ}_c = \Delta F'^{\circ}_1 + \Delta F'^{\circ}_2 + \Delta F'^{\circ}_3.$$

$$\text{thus:} \quad \Delta F'^{\circ}_3 = \Delta F'^{\circ}_c - \Delta F'^{\circ}_1 - \Delta F'^{\circ}_2$$

$\Delta F'^{\circ}_1$ can be taken from the value of Borsook[16] for E'° (—0.28 volt at pH 7) of this equilibrium. When corrected for pH 7.8, this gives, in the endergonic direction, + 13,500 calories. $\Delta F'^{\circ}_2$ can be calculated only tentatively with the help of not too accurate thermal data according to the equation: $\Delta F_2 = \Delta H_2 - T\Delta S_2$. In general, ΔH refers to the difference of the heat content of the pure substances. The subtrahend should include, besides the difference of the entropies (S) of the pure substances, the difference of the entropy changes in dissolving the substances at standard conditions (molar concentration, pH = 0). The terms for the ionization of the acid formed by oxidation and for shifting the pH from 0 to the pH of the equilibrium (7.8) finally must be added. In our cases, however, ΔH_2 corresponds to the difference of the heat content of the dissolved diluted substances, since only the heats of reaction of the dissolved phosphorylated compounds are known and not their heats of combustion. It would be entirely misleading to use instead of this the combustion heats of the unphosphorylated compounds without a thorough and critical evaluation. The combustion heat of the dimeric racemic glyceraldehyde[17] cannot be reconciled with other heat measurements on trioses, as was pointed out earlier.[19] The combustion heat of dioxyacetone[18] with the addition of the heat of solution is in better agreement with the value calculated from dissolved fructose and the endothermic reaction of the zymohexase : fructose-1, 6 diphosphate $\leftrightharpoons$ 2 dioxyacetonephosphate. Since the isomerisation of the triosephosphates is thermoneutral ($\Delta H > 1000$ calories), the value of H for dissolved glyceraldehyde may then be compared with the calculated value of dissolved glyceric acid.[1] Other possible calculations are based on the measured heat of the dismutations : triosephosphate + acetaldehyde = phosphoglycerate + ethyl alcohol; and triosephosphate + pyruvate = phosphoglycerate + lactate.[12, 20] In these cases the difference of the heat content of dissolved acetaldehyde and ethyl alcohol, and likewise of pyruvic and lactic acid must be subtracted. These values can be taken from known combustion heats and heats of solution and dilution. Moreover, one must take into account the concomitant phosphorylation of hexose in the first mentioned enzymatic dismutation, and of creatine in the second one, as well as the heat of neutralization. In the mean, $\triangle H$ for the reaction glyceraldehydephosphate + $H_2O \rightarrow$ phosphoglycerate + $2H^+$ turns out to be + 4000 calories (± 1500 calories).* $T\triangle S$ for glyceraldehyde (phosph)/glyceric acid (phosph) in their pure state probably is small, judged from analogies in the table of Parks and Huffman[21] (table 40, p. 210). The possible differences of the change of entropy by solution must be disregarded owing to lack of suitable data.† Thus the $T\triangle S$ value, which has to be taken into account, is that of the molecule of water which disappears: $T\triangle S_{(H_2O)} = \triangle H_{(H_2O)} - \triangle F_{(H_2O)} = -68{,}300 + 56{,}600$ calories $= -11{,}700$ calories. The ionization of glyceric acid to glycerate at pH 0 is endergonic. The pK of the glyceric acid group in phosphoglyceric acid = 3.42.[22] Since $-RT\ln K = -1342 \cdot (-3.42) = +4600$ calories at 20° C, $\triangle F^{\circ}_2$ for glyceraldehydephosphate + $H_2O \xrightarrow[-3H^+]{-2e}$ phosphoglycerate$^-$ = $+4000 - 11{,}700 + 4600$ calories $= -3100$ calories.

The shift of the system of pH 0 to pH 7.8 follows at 20° C, a 58 millivolt slope, as far as pH 3.42 and an 87 millivolt slope from pH 3.42 to 7.8. This corresponds to $-27{,}000$ calories. Thus, $\triangle F'^{\circ}_{2\,(pH\ 7.8)} = -3100 - 27{,}000$ calories $= -30{,}100$ calories. No correction is applied for the increase in strength of the second phosphate group of phosphoglycerate compared with glyceraldehydephosphate (pK 5.98 instead of pK 6.75),[22] which possibly would add − 1000 calories.

Inserting the $\triangle F$ values in the complete equation we get: $\triangle F'^{\circ}_{2} = -4700 - 13{,}500 + 30{,}100$ calories $= +12{,}000$ calories. In the exergonic direction ($ATP \rightarrow ADP + H_3PO_4$), $\triangle F'^{\circ}_{2} = -12{,}000$ calories.

---

* For this calculation, the heat of formation of the glyceraldehyde group (dissolved) is taken as − 140,700 calories, of water as − 68,300 calories and of the glyceric acid group (diss.) as − 205,000 calories. The values of $\triangle H_2$ obtained by the four different methods of calculation are:

From dioxyactone (diss.), glyceric acid (diss.), and thermoneutrality of the isomerisation,[19a] + 3500 calories.

From fructose (diss.) → hexosediphosphate → 2 triosephosphate, glyceric acid (diss.),[19a] + 5300 calories.

From the dismutation with acetaldehyde/alcohol,[12] + 5000 calories.

From the dismutation with pyruvate/lactate,[20] between + 2500 and 4500 calories.

† From $K_{isomerase} = 22$ for glyceraldehydephosphate → dioxyacetone phosphate it follows that $\triangle F$ of this isomerisation equals − 1800 calories (in dilute solution). But this equilibrium does not enter the computation in the text.

This value is in good agreement with the value for $\triangle F'^{\circ}$ obtained by Dr. Lipmann for the dephosphorylation of phosphopyruvic acid, namely, — 11,250 calories.

It is worth while to compare this total equilibrium of the coupling reaction with the intermediate equilibrium found by Warburg and Christian in the presence of cozymase and pure oxidizing enzyme:

$$K_{\text{oxidation}} = \frac{\text{DPNH} \times 1,3\text{-diphosphoglyceric acid}}{\text{DP}\overset{+}{\text{N}} \times 3\text{-glyceraldehydephosphate} \times H_3PO_4}$$

This equilibrium constant is strongly dependent on the pH, and at 7.8 and 20° C probably is about 3, only 1/1000 of that of the total equilibrium. Therefore, $\triangle F'^{\circ}$ for glyceraldehydephosphate + $H_3PO_4 \rightarrow$ 1, 3 diphosphoglyceric acid is about — 14,000 calories, since $\triangle F'^{\circ}$ for the oxidoreduction with DPN is only some 100 calories. We can deduce from this result that the consecutive reaction: 1, 3-diphosphoglyceric + ADP→ 3-phosphoglyceric + ATP, must have a $\triangle F'^{\circ}$ of about — 4000 calories in order to obtain the $\triangle F'^{\circ}$ of the coupling reaction. Such a value, corresponding to a log K of 3 for the transphosphorylation would mean, that, with equal concentrations of the reactants, 3–4 per cent of diphosphoglyceric acid and ADP are in equilibrium with 96–97 per cent of 3-phosphoglyceric acid and ATP. Although it was announced at one time from Warburg's laboratory that the enzyme in question was isolated, nothing is known so far to verify this computation. I had, however, concluded from experiments on dephosphorylation, where this reaction was involved, that the equilibrium would be far to the right.[29]

Let us now consider briefly the implications of the high phosphate-bond energy liberated by splitting off of phosphate, or absorbed by uptake of phosphate. In the oxidoreduction step, nearly as much of the free energy of the oxidation of glyceraldehyde to the glyceric acid level is taken up by phosphorylation of ADP to ATP, as is taken up for the reduction of DP$\overset{+}{\text{N}}$ to DPNH. The former is a net gain in free energy, while the dihydropyridine is reoxidized in the reduction of one mole of pyruvic to lactic acid. The free energy change of this reduction (pyruvate to lactate) at pH 7 is about + 8300 calories. With a small loss in free energy, the reoxidation of dihydrocozymase reconverts the product of oxidation to lactic acid, that is, to the oxidative level of glucose.

Nature is still more skillful in accumulating the potential energy of glycolysis in phosphate-bond energy. The oxidation to glyceric acid makes possible the formation of the second energy-rich phosphate bond, which is created by the dehydration of the intermediate 2-phosphoglyceric acid, to phosphopyruvic acid. This was already discussed some years ago by Lipmann.[1] The total heat change from glycogen to lactic acid amounts to 16,500 calories per mole of lactic acid without neutralization, or to 18,000 calories, with neutralization by ordinary buffer substances (like bicarbonate or phosphate). The creation of two energy-rich phosphate bonds would mean a gain in standard free energy of about 24,000 calories. This amount is about 30 per cent more than the total heat change—a very remarkable, but not miraculous result. Dean Burk had calculated, many years ago, that lactic acid formation in muscle under the conditions prevailing in the living organ could yield 50 to 80 per cent more free energy than total heat.[24]

In the living muscle, two moles of creatinephosphate can be synthesized for one mole of lactic acid formed, which corresponds to two energy-rich phosphate bonds per mole. We identify the steps involved as the transfer of the carboxyl phosphate of 1, 3-diphosphoglyceric acid and of the phosphate group of phosphopyruvic acid. Indeed, starting with hexosediphosphate, one easily obtains in enzyme extracts the synthesis of two moles of creatinephosphate per mole of lactic acid, where the over-all reaction is slightly endothermic and the energy available in the glycolytic splitting (18,000 calories per mole of lactic acid) is accumulated in the phosphate bonds of the two phosphocreatine molecules.[24] Nevertheless, the difficulty remains to be explained as to how, in the living muscle, hexosediphosphate has gotten its two phosphates. One phosphate group surely arises by means of the Cori reaction in the sequence: Glycogen + phosphate → glucose-1-phosphate → glucose-6-phosphate → fructose-6-phosphate. But in the muscle extract, fructose-6-phosphate is phosphorylated to fructose-1, 6-diphosphate only by means of the adenylic system, by consuming or wasting an energy-rich phosphate bond. Therefore, for one mole of hexose split, out of four energy-rich phosphate bonds, only three are completely comprehensible. If a reaction of the type, 2 fructose-6-phosphate → 1 hexosediphosphate + 1 hexose, should occur, the difficulty in explaining the creation of the fourth phosphate bond would be overcome. But such a reaction is, so far, not known.

Without doubt, the formation of the carboxyl phosphate in 1,3-diphosphoglyceric acid is responsible for the autocatalytic formation of hexosediphosphate during the so-called phosphate period of cell-free alcoholic fermentation. Every molecule of hexosediphosphate which ferments not only regenerates a new one by means of its original content of two phosphate groups, but it also regenerates two by means of this extra phosphate taken up during oxidation. The result corresponds to the snow-ball collection practice, formerly used in promoting charitable enterprises, where every contributor not only has to pay for himself, but, at the same time, to get a second man to pay the same amount and to agree to do likewise. How this "autocatalysis" is brought about in an enzyme extract is completely obvious now, but it is still a matter of controversy as to what, in the living yeast, controls the synchronization of phosphorylation and dephosphorylation, at least after a short initial period, in which, after addition of sugar, most of the inorganic phosphate in the cell is esterified. This controlling factor, apparently, is damaged or destroyed by killing the yeast.

In discussing this problem, I shall at first briefly mention the concepts of Nilsson, in Stockholm,[25] who still in 1942,[26] in a lecture which he gave during the present war in my former Institute in Heidelberg, deemed it especially suitable to attack my scheme of fermentation and to cling to his own old idea:—that sugar is not at all fermented by way of hexosediphosphate, but exclusively by way of hexosemonophosphate. The latter then should break down into one phosphorylated and one unphosphorylated triose molecule. In the living yeast, both parts would ferment; in extracts, only the unphosphorylated halves, and the phosphorylated portions would return to hexosediphosphate through the action of aldolase. A lipoid structure in the living cell would be responsible for the fermentation of the phosphorylated triose and this structure would be damaged or destroyed by killing the yeast. The author inferred these assumptions from fermentation curves which he obtained from several types of dried yeast:—either the fermentation of sugar goes to completion with smoothly decreasing speed, or a break occurs after half of the sugar is fermented. This latter situation is the expression for the known equation of Harden and Young. In this case, provided there is less sugar present than its equivalent of inorganic phosphate, after fermentation of half of the added sugar, the other half is completely esterified to hexosedi- and monophosphate.

Some time ago, Warburg and Christian[27] backed this scheme of Nilsson's, but later, albeit tacitly, adopted my scheme. I think that it is not necessary to consider Nilsson's concept seriously in view of the bulk of the evidence that hexosediphosphate is the indispensable thoroughfare in the course of sugar breakdown. For those who are still intrigued by the slow fermentation of hexosediphosphate in yeast extracts in comparison with that of sugar, I mention only two sets of experiments which give a clue to this behavior. If one adds creatine to the maceration juice of yeast together with the phosphorylating enzyme from muscle, hexosediphosphate ferments to alcohol and carbon dioxide, at the same speed at which sugar ferments in the same extract. Creatine, which is foreign to yeast, serves now as the phosphate acceptor.[24, 28] In a second type of experiment, arsenate may be added in about millimolar concentration. Under otherwise favorable conditions, hexosediphosphate here again ferments as rapidly as does sugar. In this case we assume, as do Warburg and Christian, that the coupling with phosphate uptake is interrupted by the formation of 1-arsenyl-3-phosphoglyceric acid, which splits off its arsenyl group without enzymes. I have shown recently how this explains, not only the rapid formation of phosphoglyceric acid, but also its rapid dephosphorylation.[29]

There can be no doubt that sugar ferments completely by way of hexosediphosphate in the living yeast, as well as in the non-living yeast. Long ago, I had proposed tentatively as an explanation for the different kinetics in both cases, that the adenylpyrophosphatase is the most sensitive enzyme of the fifteen to twenty different partial enzymes of fermentation which form the zymase complex. By extracting or drying the yeast, it would be damaged more extensively than the others.[30] If this is true, one can assume that the adenylpyrophosphatase in the living yeast is sufficiently active for the regeneration of adenylic acid from adenosinetriphosphate, at the same speed at which hexosediphosphate arises anew from the oxidative coupling reaction with phosphate transfer to glucose. In this way, formation and dephosphorylation of hexosediphosphate would be synchronized. I admit that, so far, this idea is not definitely proved.* We shall, therefore, consider two other possibilities. Through the regeneration of hexosediphosphate, the energy-rich phosphate bond of adenosinetriphosphate is wasted. In the metabolism of muscle, this situation is avoided, because creatine takes up the energy-rich bond and stores it in the form of creatinephosphate. A continuous supply of energy-rich phosphate bonds is probably needed for many other synthetic purposes in the cell metabolism, as, for instance, for the formation of thiamindiphosphate from thiamin. The yeast cell and all other cells may contain very many such phosphate acceptors which, in a stationary state, would finally release the phosphate again by means of phosphatases, while, during growth, part of it would be preserved. I mention here briefly the related case of the autotrophic sulfur bacteria, where, according to the highly interesting discovery of Umbreit, Vogler, le Page,[31, 31a] the energy of the sulfur oxidation is stored in adenosinetriphosphate and can be used anaerobically in the dark for the assimilation of carbon dioxide. Generally, synthesis may be mediated in this manner. This extra supply of energy-rich phosphate bonds in the stationary state of fermentation may, therefore, be of the greatest importance in nature. Probably such a mechanism is combined with the first-mentioned, and the adenylpyrophosphatase releases only the excess of that high energy phosphate not otherwise needed. Finally, the third possibility remains, that the living yeast makes use of a special enzyme for dephosphorylation of the carboxyl phosphate. This enzyme then would be destroyed or damaged by the extraction procedures. Such an enzyme is not known so far, but, if it exists, it would waste the energy-rich bond completely. Probably it would function similarly to the adenylpyrophosphatase in the last mentioned case. That this latter enzyme is preferentially and primarily injured by killing the yeast, is, after all, the most probable of these explanations.

---

* I will mention, however, recent experiments which I performed to prove this point. We destroyed the yeast by freezing in liquid air or by ultrasonic vibration. The fermentation of hexosediphosphate by the extract of the cell fragments is increased many times by the addition of purified adenylpyrophosphatase from potatoes (**O. Meyerhof.** Jour. Biol. Chem. In press).

When we look over the whole picture of the biological phosphorylation in carbohydrate breakdown, we arrive at some generalizations. I may cite some of these: Energy-rich phosphate bonds are only created, directly or indirectly, by the oxidative reaction steps in phosphorylated intermediates. On the other hand, all true ester phosphates, where the phosphate esterifies alcoholic groups, arise exclusively from transphosphorylation with the adenylic system or by intramolecular phosphate shift. Inorganic phosphate is never taken directly into alcoholic groups, but only into carbonyl or carboxyl groups. Probably the following generalization is also true: the function of the adenylic system as a dissociable coenzyme consists only in transphosphorylations, not in uptake or direct release of inorganic phosphate. This statement seems to contradict the important recent discoveries of Cori,[32, 32a] that adenylic acid is a prosthetic group in the phosphorylase. The mechanism of this reaction, in spite of the abundance of discoveries made by Cori and his group, is, so far, not completely understood. Since adenosinedi- and triphosphate cannot replace the adenylic acid in the phosphorylase reaction, its function here must be different; moreover, it seems from the latest statements of Dr. Cori[33] that it may form a compound with another group, perhaps a dinucleotide, so that, in principle, the last generalization may also hold.

I have purposely dealt here only with what I called the first and second historic period, leaving to Dr. Kalckar to discuss the present-day problems. These, indeed, are of more acute interest. On the other hand, much experimental material of the past years is still available for profitable interpretation and evaluation. A conference such as ours can promote such critical examination and coordination of thermal, electrical and chemical measurements, as may be necessary for drawing thermodynamic conclusions.

BIBLIOGRAPHY

1. **Lipmann, F.**
1941. Advances in Enzymology **1**: 99.

2. **Kalckar, H. M.**
1941. Chemical Reviews **28**: 71.

3. **Colowick, S. P., H. M. Kalckar, & C. F. Cori**
1941. Jour. Biol. Chem. **137**: 343.

4. **Ochoa, S.**
1941. Jour. Biol. Chem. **138**: 751.
1943. Jour. Biol. Chem. **151**: 493.

5. **Lohmann, K.**
1937. Angewandte Chemie **50**: 97.

5a. **Lundsgaard, E.**
1930. Biochem. Z. **217**: 162.

6. **Nachmansohn, D.**
1928. Biochem. Z. **196**: 73.
1929. Biochem. Z. **208**: 237.

7. **Dubuisson, M., & W. Schulz**
1938. Plügers Arch. f. Physiol. **239**: 776.

7a. **Dubuisson, M.**
1940. Arch. internat. physiol. **58**: 203.

8. **Meyerhof, O.**
1930. Chemische Vorgänge in Muskel. J. Springer. Berlin.

8a. **Meyerhof, O.**
1941. Biol. Symposia **3**: 239.

9. **Meyerhof, O., & Nachmansohn, D.**
1930. Biochem. Z. **222**: 1.

10. **Belitzer, V. A., & E. T. Tzibakowa**
1939. Biokhimia **4**: 516.

11. **Meyerhof, O.**
1941. Biol. Symposia **5**: 141.

12. **Meyerhof, O., P. Ohlmeyer, & W. Möhle**
1938. Biochem. Z. **297**: 113.

13. **Warburg, O., & W. Christian**
1939. Biochem. Z. **303**: 40.

14. **Negelein, E., & W. Brömel**
1939. Biochem. Z. **303**: 132.

15. **Meyerhof, O., & R. Junowicz-Kocholaty**
1943. Jour. Biol. Chem. **149**: 71.

16. **Borsook, H.**
1940. Jour. Biol. Chem. **133**: 629.

17. **Neuberg, C., Hofmann, & M. Kobel**
1931. Biochem. Z. **234**: 341.

18. **Kobel, M., & W. Roth**
1928. Biochem. Z. **203**: 159.

19. **Meyerhof, O., & K. Lohmann**
1935. Biochem. Z. **275**: 430.

19a. **Meyerhof, O., & W. Schulz**
1936. Biochem. Z. **289**: 87.

20. **Meyerhof, O., & W. Schulz**
1935. Biochem. Z. **281**: 292.

21. **Parks, G. S., & H. M. Huffmann**
1932. The free energies of some organic compounds. Chemical Catalogue Comp. New York.

22. **Kiessling, W.**
1934. Biochem. Z. **273**: 103.

23. **Burk, D.**
1929. Proc. Roy. Soc. (**B**)**104**: 153.

24. **Meyerhof, O., W. Schulz, & Ph. Schuster**
1937. Biochem. Z. **293**: 309.

25. **Nilsson, R., & F. Alm**
1936. Biochem. Z. **286**: 254, 373.
1940. Biochem. Z. **304**: 285.
1941. Biochem. Z. **308**: 262.

26. **Nilsson, R.**
1943. Naturwiss. **31**: 25.

27. **Warburg, O., & W. Christian**
1936. Biochem. Z. **287**: 291.

28. **Meyerhof, O., W. Kiessling, & W. Schulz**
1937. Biochem. Z. **292**: 25.

29. **Meyerhof, O., & R. Junowicz-Kocholaty**
1942. Jour. Biol. Chem. **145**: 443.

30. **Meyerhof, O.**
1937. Erg. der Physiol. **38**: 10, 54.

31. **Vogler, K. G., & W. W. Umbreit**
1942. Jour. Gen. Physiol. **26**: 157.

31a. **Le Page, G. A., & W. W. Umbreit**
1943. Jour. Biol. Chem. **147**: 263.

32. **Cori, G. T., S. P. Colowick, & C. F. Cori**
1938. Jour. Bil. Chem. **123**: 381.

32a. **Cori, G. T., & C. F. Cori**
1940. Jour. Biol. Chem. **135**: 733.

33. **Cori, C. F. et al**
1943. Jour. Biol. Chem. **151**: 21, 31, 39, 57.

THE FUNCTION OF PHOSPHATE IN ENZYMATIC SYNTHESES

By H. M. Kalckar
The Public Health Research Institute of The City of New York, Inc.

INTRODUCTION

In this paper an attempt will be made to give a coherent picture of what we know about the function of phosphate in the thermodynamics of biological systems. If it is possible today to draw a complete picture and correlate and harmonize all the facts which we have accumulated—and I think it is possible to do so—we can attribute it to the fact that the material furnished originates from so many diversified quarters, some having a more physical, others a more chemical background. I may illustrate this with some examples.

Determination of heat changes accompanying trans- and dephosphorylations originated from Meyerhof's laboratory.[1] Combustion data of sugars have been collected by Neuberg and his group[2] in Germany and by the California school in this country. The latter school, represented by Parks and Huffman,[3] Borsook[4] and others, has furthermore been able to give us exact entropy values for a number of important biological compounds. The determination of biological oxidation reduction potentials, which was developed by Mansfield Clark and Michaelis, and their pupils, has also been of indispensable value for our understanding of energy relationships in biological systems. Finally, I ought to mention the long series of discoveries of new phosphoric esters, starting with the sugar phosphoric esters of Harden and Robison, followed in 1927 by the discovery of phosphocreatine by Fiske and Subbarrow, and ending with the isolation of acylphosphates by Warburg, Negelein and Lipmann in 1939.

THE POTENTIAL ENERGY OF TRIOSES

It is not the idea in this lecture to give a historical development of phosphorylation, but rather to illustrate that phosphorylations are processes of essential significance for the generation of energy in the living cell. We all know that the degradation of sugars is one of the most important energy-yielding reactions which take place in biological systems. It ought to be pointed out, however, that the general formula $C_nH_{2n}O_n$ for sugars does not give us any information whatsoever about the potential energy of a compound. For instance, triose and lactic acid have both the formula $C_3H_6O_3$ but, as we shall see, the former possesses a much higher potential energy than the latter. Lactic acid is an α-hydroxy acid of relatively high stability, triose is a carbonyl-di-hydroxy structure of an extraordinarily high degree of instability. The two thermodynamically instable groups of triose are the carbonyl group, which can be oxidized to the very stable carboxylate group, and the dihydroxy structure, which by an anhydride reaction can be converted to the much more stable α-keto structure.

Before entering into a discussion of the thermodynamical role of phosphate, I think it is worth while for a moment to leave the phosphate out of the picture and see what would happen to the potential energy of the triose if this substance were converted to free pyruvic acid directly. The step glyceraldehyde $+ H_2O \rightarrow$ glycerate$^-$ $+ 2H^+ + 3E$ has already been discussed by Dr. Meyerhof, so only a few remarks are necessary. Although this step is only slightly exergonic at pH 0, i.e., when it is calculated from heats of formation under standard conditions, it is one of the strongest exergonic reactions known to occur in biological systems at pH 7. We know that when 2 hydrogen ions are liberated per 2 electrons the normal potential changes 60 millivolts (mv.) per pH unit. In this case, however, a third hydrogen ion is liberated, due to the formation of a carboxyl group. The pK of glyceric acid is around 3.4. At a more alkaline pH the acid is therefore practically completely dissociated and, consequently, the third hydrogen ion only affects the pH slope of the oxidation reduction potential between pH 0 and pH 3.5. This means that between pH 3.4 and pH 7 the oxidation reduction potential (E'_0) changes 90 mv. per pH unit, or, altogether 324 mv., and between pH 0 and pH 3.4, four times 60 mv. or 204 mv. The total change is therefore 528 mv. or approximately −25,000 calories. Since the conversion of glyceraldehyde $+ H_2O$ to glycerate at pH 0 has a ΔF of −4000 calories,[5] the ΔF of this reaction at pH 7 is −29,000 calories and at pH 8, around −33,000 calories. Very few biological oxidations with the exception only of other carbonyl oxidations do release such large amounts of free energy. It may be profitable to compare the ΔF of the reaction with that of other biological oxidations. The system lactate $\rightleftharpoons$ pyruvate $+ 2H^+ + 2E$ has, according to Barron and Hastings,[6] an E'_0 at pH 7 of −180 mv. which corresponds to −8300 calories. Ball[7] found the E'_0 at pH 7 of the pyridine nucleotide system to be −280 mv. which corresponds to −12,500 calories, i.e., still more than 15,000 calories short of the glyceraldehyde-glycerate-system, a fact of considerable importance as will appear later. The very high relative stability of the carboxylate group will also be discussed later.

The other important energy-yielding step, the formation of the anhydride pyruvic acid from the dihydroxy acid, glyceric acid, has recently been discussed by Lipmann[8] in an interesting analysis. Lipmann has collected thermal data from various sources and has shown that the dehydration of glycerate to pyruvate liberates as much as 8000 calories per mole. Almost 2000 calories of this amount are derived from an increase in entropy due to the formation of a C =O group. The corresponding conversion of glyceraldehyde to methylglyoxal must give rise to approximately the same increase in entropy since the same structural changes are involved. The △ H based on experimental combustion data, however, seems to be considerably smaller. The heat of combustion of methylglyoxal is, according to the careful determinations of Neuberg and Hofmann,[9] 345,700 calories per mole; that of dihydroxy-acetone, according to Kobel and Roth,[10] 343,100 calories. The heat of combustion of glyceraldehyde is presumably very nearly the same or, if anything, a little higher. The value, 338,000 calories, found by Neuberg, Hofmann and Kobel[2] applies only to the di-molecular solid glyceraldehyde. The mono-molecular glyceraldehyde has probably a combustion heat approximating 345,000 calories, judging from the equilibrium constant of the reaction phospho-glyceraldehyde $\rightleftharpoons$ phospho-dihydroxyacetone.[11] Thus, the △ F of the reaction glyceraldehyde $-$ $H_2O \rightarrow$ methylglyoxal cannot be larger than $-$ 2000 calories at most. It may also be of interest to mention, at this point, that the calculated heat of combustion ad modum Kharasch[12] does not check at all with the observed heats of combustion of methylglyoxal and dihydroxyacetone, but yields values 8000 to 15,000 calories too high.

The oxidation of methylglyoxal (α-keto aldehyde) to pyruvic acid (α-keto acid) has a △ F of about $-$ 27,000 calories at pH 7. If the free energy released by converting triose into pyruvate or lactate were simply scattered as heat, the conversion could follow two different pathways. The triose could first be dehydrated and then oxidized, or vice versa. The first pathway is that proposed by Neuberg:[13] the anhydride of triose, methylglyoxal, is first formed, and this compound is then subsequently oxidized to pyruvate, or, by an internal dismutation, converted into lactate. Formation of methylglyoxal from triose is a non-enzymatic reaction, whereas conversion of methylglyoxal to lactate is catalyzed by a specific enzyme, the so-called methylglyoxalase.[14] Formation of pyruvate and lactate from triose through methylglyoxal may be a pathway of biological significance, although it is, for the time being, considered a process of no biological importance.

The second pathway, the study of which was initiated by Embden[15] and continued by Meyerhof and Lohmann, seems to be the most important biological process for the degradation of sugar. The triose is first oxidized to glycerate and the glycerate is then dehydrated to pyruvic acid, which subsequently oxidizes another molecular of triose, itself being reduced to lactate. Whereas lactic acid can be formed from triose through methylglyoxal in the absence of phosphate, the pathway via glyceric and pyruvic acids requires phosphate and phosphorylated products.

Time will not permit me to go into any detail as to why the Embden scheme has been adopted as the basis for our concept of fermentation. The chemical synthesis of the phosphotrioses by Fischer and Baer,[16] which made large scale experimentation with these compounds possible, had an important bearing upon the establishment of that scheme. The enzymatic studies of Meyerhof and Kiessling[17] finally consolidated the Embden scheme.

THE RELATION OF PHOSPHORYLATIONS TO THE PRESERVATION OF POTENTIAL ENERGY

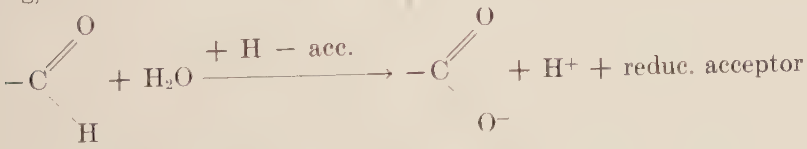
When we discuss the role of phosphate in carbohydrate metabolism, it is important to distinguish between the first steps in sugar oxidation (steps which are common to those of fermentation) and the further steps of oxidation (and decarboxylation) of the acids formed from the sugar acids.

If rather special types of glucose oxidation are excluded, e.g., oxidation by glucose oxidase or by Robison ester oxidase, we shall see that phosphate plays a double role, so to speak, in the conversion of sugar to pyruvic acid. That is, only phosphorylated sugars are metabolized, and inorganic phosphate is necessary for the oxidation of the phosphorylated sugar.

The requirement of inorganic phosphate for various carbonyl oxidations, as well as the fact that only phosphorylated sugars participate in a number of important reactions, are features of major interest in a discussion of thermodynamics of biological phenomena.

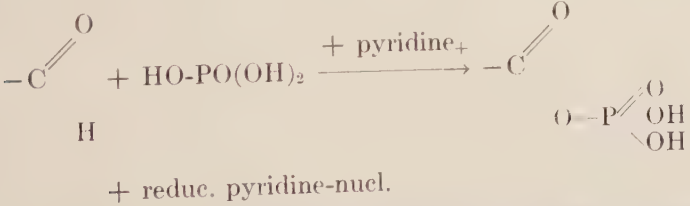
The coupling of fermentation with uptake of phosphate was first observed by Harden[18] and Young, who also were the first to discover the formation of sugar phosphate esters. The thermodynamical importance of this coupling in connection with phosphorylation of creatine was first stressed in 1930 by Lundsgaard[19] in his well-known studies of the anaerobic phosphocreatine breakdown during alactacid muscle contraction. Lundsgaard demonstrated for the first time a proportionality between the breakdown of a labile phosphate compound and mechanical work, and a resynthesis of the labile phosphate compound (phosphocreatine) coupled with lactic acid formation. The coupling between oxygen uptake and phosphorylation appears from the same studies, and was further strengthened by extract experiments of Dische,[20] Engelhardt, Meyerhof, and many others. The studies of Green, Needham and Dewan[21] as well as of Meyerhof[22] and his group showed that the coupling between the oxidation of phosphotriose and phosphate uptake is compulsory and mutual, and that the reverse reaction, the reduction of phosphoglycerate to phosphotriose, is coupled with a liberation of inorganic phosphate derived, as shown by Meyerhof, from adenosine triphosphate.

The mechanism of the coupling was explained by Warburg and his group through a number of most decisive experiments. Warburg and Christian[23] isolated and crystallized the phospho-glyceraldehyde dehydrogenase, and their collaborators, Negelein and Brömel,[24] isolated, as the primary oxidation product, di-phospho-glyceric acid as the strychnine salt. Expressed in more general terms,—what Warburg and his associates had shown was that the aldehyde group in triose is not oxidized as aldehyde *hydrate*, as suggested by Wieland and Thunberg,



$$-\overset{\displaystyle O}{\underset{\displaystyle H}{C}} + H_2O \xrightarrow{+\ H\ -\ acc.} -\overset{\displaystyle O}{\underset{\displaystyle O^-}{C}} + H^+ + \text{reduc. acceptor}$$

but as aldehyde *phosphate*,

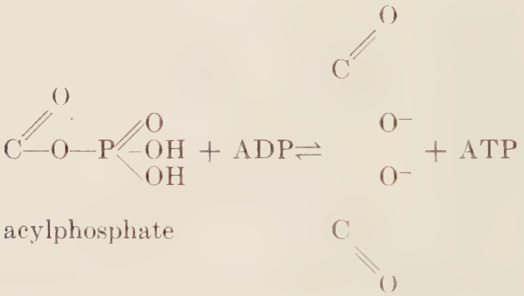


$$-\overset{\displaystyle O}{\underset{\displaystyle H}{C}} + HO\text{-}PO(OH)_2 \xrightarrow{+\ pyridine_+} -\overset{\displaystyle O}{\underset{\displaystyle O-P(=O)(OH)OH}{C}}$$

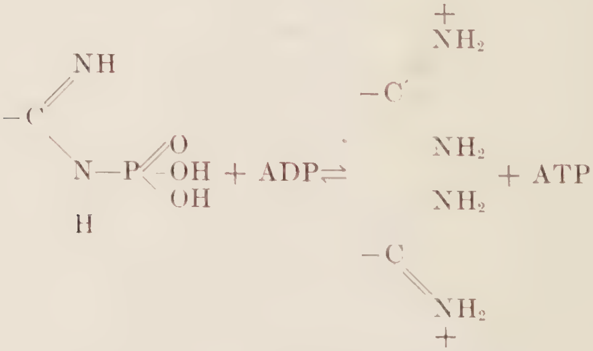
$$+\ \text{reduc. pyridine-nucl.}$$

and this reaction is reversible due to the fact that instead of the stable carboxylate ion, the unstable acylphosphate, carboxylphosphate, is formed. Lipmann[25] has been able to show that the oxidation of pyruvic acid to CO_2 and acetate follows the same pattern, at least in microorganisms. Phosphate is necessary, and Lipmann[26] has recently succeeded in isolating acetylphosphate, the primary oxidation product. His most recent experiments indicate that also this reaction may be reversed.

Bücher,[27] in Warburg's laboratory, has isolated and crystallized another enzyme which catalyzes the equilibrium reaction: Diphosphoglyceric acid + adenosine diphosphate ⇌ Monophospho-glycerate + adenosine triphosphate, or in more general terms: Acylphosphate + ADP ⇌ carboxylate⁻ + ATP.

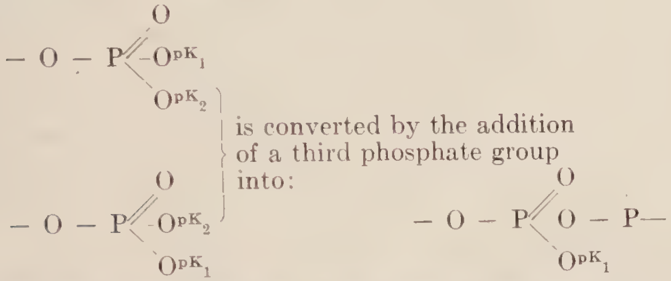


This equilibrium has an astonishing resemblance to that described by Lohmann, and later by Lehmann[28] in 1935:



Phospho-amidine + ADP⇌Amidine + ATP

Two mesomeric structures and a new pyrophosphate are formed in both cases. The same changes in degrees of mesomerism (resonance) apply to phosphate group No. 2 of adenosine diphosphate. This second group might be assumed to display a kind of mesomerism which could be pictured as follows:



is converted by the addition of a third phosphate group into:

It is a well-established fact that mesomerism, particularly of that symmetrical type described here, contributes to a great extent to the stability of a group.[29, 30, 31] This applies, too, for free radicals, a fact which has been exploited so successfully by Michaelis.[32] If a group of mesomeric character is phosphorylated, the symmetry disappears completely and thus the stability decreases greatly, which again means that the potential energy increases. The carboxylate group of phospho-glycerate or of acetate, the amidine group of creatine or arginine, the phosphate group of adenosine diphosphate, are all mesomeric structures which, upon phosphorylation, lose, more or less, their symmetry, and thus their mesomerism, and acquire instead what the structural chemists call "opposing resonance" (Coryell, cf.[33]). All three types of phosphate esters just mentioned possess therefore a comparatively high potential energy. The important calorimetric studies of Meyerhof, Lohmann and Schulz give us an idea of the magnitude of the potential energy of these phosphate compounds. A determination of the △ H of the splitting of phosphocreatine or adenosine triphosphate was undertaken soon after they were isolated by Fiske and Subbarow and was found to be 11,000–12,000 calories per mole P liberated.[2, 34]. The free energy of phosphocreatine and phosphoglyceryl linkages must be close to that of the pyrophosphate in adenine nucleotides because of the readily reversed equilibria just mentioned.

The two pyrophosphate linkages of adenosine triphosphate must possess the same potential energy since the equilibrium constant of the reaction 2 adenosine diphosphate ⇌ 1 adenosine *tri*phosphate + 1 adenylic acid[35] is very nearly 1. Thus, each of these linkages represents an amount of potential energy of at least 10,000 calories.

It was mentioned before that the △ F going from carbonyl to carboxylate$^-$ at pH 7 is – 29,000 calories. The △ F of the reduction: pyridine-nucleotide ⇌ reduc. pyridine nucleotide is approximately

+ 12,500 calories at pH 7. Thus, of the — 29,000 calories generated, only 12,500 are used for the reduction and the remaining - 16,500 calories are available for synthesis. Approximately —10,000 to 12,000 calories are stored either as acylphosphate, or more permanently as pyrophosphate or amidine phosphate energy. At pH 8 the energy balance is as follows: Reduction of pyridine nucleotide* + 13,750 calories; oxidation of glyceraldehyde - 33,000 calories. Thus, — 20,000 calories are available for synthesis at pH 8. In the back reaction the reduction of acid to aldehyde, which represents the most important and most expensive reaction in the synthesis of sugar from organic acids, the pyrophosphate linkage is sacrificed for the phosphorylation of the carboxylic group. The comparatively unstable acylphosphate is then readily reduced to carbonyl. The uptake of CO_2 in Lipmann's reaction may require more energy than the reduction of the carboxyl group of glyceric acid. However, Lipmann has recently reported that there are indications that also the oxidative carboxylations can be reversed.

Lipmann's discovery of acetylphosphate aroused a good deal of interest in other oxidative decarboxylations. Banga, Ochoa and Peters showed that phosphate is necessary for the oxidation of pyruvate in brain tissue. Ochoa[36] has recently found that phosphate is also required for the oxidation of α-ketoglutarate to succinate and CO_2 If we turn our attention to oxidations other than carbonyl oxidations, it appears that they also are able to furnish energy for phosphorylations. Thus, Colowick, Welch and Cori[37] showed that the step succinate-fumarate can give rise to phosphorylation of glucose. Moreover, oxidation of fumarate or malate gives rise under certain conditions, to formation of phospho-enol-pyruvate.[38] Whether phosphate is taken up by the fumaric acid forming phosphomalic acid, or whether malate or oxalacetate is phosphorylated by a phosphate donor is not known yet. The E'_0 of the malate-oxalacetate system is around — 180 mv., which means that if the flavine system transfers electrons the span between the potentials will amount to about 4500 calories.

If one compares the oxygen consumption with the uptake of phosphate, one is almost forced to assume that the higher oxidation steps too are coupled with uptake of phosphate. Ochoa,[39] as well as Belitzer and Tsibakova,[40] for instance, finds an average of 3 phosphate ester linkages per atom of oxygen, or 36 atoms of phosphate taken up per mole sugar, converted into CO_2. Dr. Ball has already mentioned the possibility that extra phosphorylation could be generated by the passage of hydrogen from one co-enzyme to another, and has particularly called attention to the possible role of the alloxazine ring. The reduced alloxazine ring, particularly the C:O group in the 4-position, may be a point of entrance for the phosphate. In the oxidized alloxazine, the C:O groups form a conjugated pair of double bonds with the adjacent nitrogens. The mesomerism of these conjugated double bonds would cease to exist if phosphate groups were tied to the C:O groups.

---

* This reduction involves two electrons but only one hydrogen ion.

If we now turn our interest to the phosphate esterified to the sugar, we realize soon that this problem has two aspects. The first is the phosphorylation of the alcohol group in the 6-position, which is of particular importance for the degradation of the dihydroxy structure; the other, the phosphorylation of the aldehyde group forming the 1-phosphoglucose or Cori ester, which is of importance for the formation of polysaccharide.

The fate of the alcohol phosphate ester linkage is of considerable interest in a discussion dealing with thermodynamics. Most important in this connection is the final conversion of the di-alcohol-ester into an enolic-ester linkage. Meyerhof and Lohmann[41] found that one specific enzyme converts 3-phosphoglycerate into 2-phosphoglycerate where the phosphate group is on the carbon No. 2. They isolated another enzyme called enolase, which converts the 2-phosphoglycerate into the anhydride, phospho-enol pyruvate. They found, furthermore, that the latter ester has a high potential energy since it is capable of phosphorylating adenosine diphosphate to adenosine *tri*phosphate. Correspondingly, the $\triangle$ H of the phosphate splitting is in the neighborhood of 10,000 calories.[42] I have already mentioned the interesting calculations by Lipmann[8] who showed that the dehydration from a dihydroxy structure to an α-keto structure is a strongly exergonic process. He calculated that $\triangle$ F of the reaction phospho-enol-pyruvate = pyruvate + phosphate amounts to 11,250 calories/mole.

The relatively high stability of the keto acid structure as compared to the dihydroxy structure must at least partly be ascribed to the tautomeric shift between the keto and the enolic structure. Just like the mesomeric oscillations are prevented by esterification, so is the tautomeric shift. Curiously enough, it has, so far, not been possible to demonstrate any phosphorylation of pyruvate by adenyl pyrophosphate. The reason for that remains obscure.

The role of phosphate in the synthesis of polysaccharides from monosaccharide has been revealed through the studies of Cori et al. They isolated in 1936,[43] as the primary product of polysaccharide splitting, a new glucose phosphate completely different from Robison ester, in that it was extremely acid labile and that it had no reducing power. The structure of glucose-l-phosphate was established by Cori, Colowick and Cori[44] by synthesizing the ester. The formation of polysaccharide from 1-ester was indirectly observed by Schäffner, but decisively established by Cori, Cori and Schmidt[45] in this country and by Kiessling[46] in Germany. The recent studies of Cori, Cori and Green[47] further clarify several points, particularly why glycogen is necessary to initiate the reaction. Their formulation of the equilibrium is: glucose-1-phosphate + terminal glucose (of polysaccharide) $\rightleftharpoons$ new maltosidic linkage + phosphate. Doudoroff[48] and his coworkers showed that the disaccharide, sucrose, is synthesized according to the same pattern. He found the $\triangle$ F of the sucrose synthesis to be + 1700 calories at pH 6.6. We meet here the same feature as we did when the carbonyl oxidations were discussed, namely, that a reaction which proceeds irreversibly, if water is involved, becomes reversible when water is replaced by phosphate. Thus, in the irreversible splitting of poly- or disaccharides by digestion enzymes, the glucosidic linkage is split by water; in the intracellular reversible splitting, the glucosidic linkage is broken by phosphate.

If we want to sum up very briefly what we actually have learned about the function of phosphate in biological syntheses, we may do it as follows. In the oxidation of sugars, the potential energy of the carbonyl group and the dihydroxy structure is transformed into energy rich phosphate linkages.

The next question is: What is the significance of the energy rich phosphate linkages? We know that adenylpyrophosphate is a powerful phosphorylating agent, particularly towards sugars and glycerol. Thus, adenylpyrophosphate initiates the carbohydrate metabolism, be it oxidative breakdown or polysaccharide formation, by phosphorylating hexoses. We have also seen how adenylpyrophosphate by phosphorylation of a carboxyl group can initiate the reduction of acids to aldehydes.[21, 22] The existence of adenosine triphosphatase in myosin preparations, as described by Engelhardt and Ljubimowa, may be of significance for our understanding of how chemical energy is transformed into mechanical energy. However, only a vast extension of our knowledge of the chemistry of myosin will permit further conclusions in this direction.

ENERGETIC COUPLING WITH THE UPTAKE OF ALCOHOLS, SUGARS AND AMINO COMPOUNDS

I have not attempted, in this survey, to make a detailed account of all the numerous biological reactions in which phosphate is involved, but have rather presented the salient facts in the hope that they may provide some insight into the nature of coupled reactions in biological syntheses. This brings us to the question whether phosphate is involved in every biological synthesis or whether other compounds can react in a similar way. It is important to bear in mind that carbonyl groups can react to form more or less labile complexes with a large number of substances. If such a carbonyl complex were oxidized or polymerized we should have another example of a coupled synthesis. The model experiments of Baer[49] furnish particularly striking examples of such coupled syntheses. Baer found that the oxidation of α-keto compounds by lead tetra-acetate required the presence of hydroxyl furnishing groups such as water, phosphate or alcohols. When pyruvic acid was the substrate to be oxidized, and choline the alcohol furnishing the hydroxyl groups, the primary oxidation product was acetylcholine.

It is not unlikely that ammonia and amino groups can play a similar role as was described for hydroxyl groups. Thus, Annau[50] has reported that ammonia stimulates the oxygen consumption of tissue slices, and Örström[51] found that the respiration of unfertilized eggs is greatly increased by addition of ammonium salts. There is every reason to be on the alert for the possibility that small amounts of ammonia or alcohols might be necessary components in certain enzymatic oxidations, particularly oxidations of carbonyl compounds. The amounts of alcohols or related substances necessary for such an oxidation might very well be so small as to escape our attention, since the same alcohol molecule might be used over and over again. We have an example of such a case in the action of minute amounts of arsenate on the oxidation of glyceraldehyde. According to Warburg and Christian[23] arsenate works essentially in the same way as phosphate except that the corresponding carboxyl arsenate compound formed is so unstable that it breaks down at once, thereby liberating arsenate continuously. This explains why even traces of arsenate are able to maintain the oxidation of triose, whereas phosphate is required in much larger amounts. The finding of Warburg and Christian[52] that glucose or fructose stimulated the oxidation of phosphohexonic acid may also be of interest in this connection. They found that the oxidation of phosphohexonic acid was more complete in the presence of small amounts of the hexoses, although the latter were neither oxidized nor phosphorylated.

There is also evidence that esters other than phosphoric esters can serve as precursors in the formation of polysaccharides. This has been demonstrated most convincingly by Hehre and Sugg[53] in their interesting studies of the bacterial synthesis of dextran from sucrose. They found that the reaction proceeds according to the following formula: glucose-1-fructose (= sucrose) + terminal glucose unit (of dextran)

$$\rightleftharpoons \text{fructose} + \text{new glucosidic linkage}$$

Thus, the reaction is analogous to that discovered by Cori, differing only in that sucrose (which actually is glucose-1-fructose) takes the place of the Cori ester (glucose-1-phosphate).

There is reason to believe that reactions analogous to the enolase reaction (2-phosphoglycerate $-$ $H_2O \rightleftharpoons$ phospho-enol-pyruvate) may exist. The conversions of cysteine or serine into pyruvate, reactions which have been studied by Smythe, Fromageot, Chargaff and Binkley, offer interesting examples of this type (cf. Binkley[54]). I refer to Dr. Smythe's article[55] for further information in this regard. However, I should like to emphasize here one point of thermodynamical interest concerning the conversion of cysteine to pyruvate. If the change of free energy in this reaction should prove to be as large as that of the conversion of glycerate to pyruvate, it would imply that the removal of hydrogen sulphide from glutathione creates a powerful glycyl-glutamate donor, as the removal of water from phosphoglycerate forms a powerful phosphate donor.

It seems quite probable that the near future will witness the discovery of a number of important coupled reactions following the same pattern as those described for phosphoric esters.

REFERENCES

1. **Meyerhof, O.**
   1930. Die chemische Vorgange im Muskel. Julius Springer, Berlin.
2. **Neuberg, C., E. Hofmann, & M. Kobel**
   1931. Biochem. Zeit. **234**: 341.
3. **Parks, G. S., & H. M. Huffman**
   1932. The Free Energies of Some Organic Compounds. The Chemical Catalog Co., Inc. New York.
4. **Borsook, H., & H. M. Huffman**
   1938. Some Thermodynamical Considerations of Amino Acids, Peptides and Related Substances. Chapter XV in Schmidt's "The Chemistry of Amino Acids and Proteins." Charles C. Thomas, Illinois.
5. **Meyerhof, O.**
   1944. Ann. N. Y. Acad. Sci.
6. **Barron, E. G., & A. B. Hastings**
   1934. Jour. Biol. Chem. **107**: 567.

7. **Ball, E. G.**
1938. Biochem. Zeit. **295**: 262.

8. **Lipmann, F.**
1941. Advances in Enzymology and Related Subjects. Vol. 1. edited by F. F. Nord and C. H. Werkman. P. 99. Interscience Publishers, Inc. New York.

9. **Neuberg, C., & E. Hofmann**
1932. Biochem. Zeit. **252**: 440.

10. **Kobel, M., & W. A. Roth**
1928. Biochem. Zeit. **203**: 158.

11. **Meyerhof, O.**
1938. Bull. soc. chim. biol. **20**: 1033, 1345.

12. **Kharasch, M. S.**
1929. Bur. Standards Jour. Research. **2**: 359.

13. **Neuberg, C.**
1913. Biochem. Zeit. **49**: 502.

14. **Dakin, H. D., & H. W. Dudley**
1913. Jour. Biol. Chem. **14**: 155.

15. **Embden, G., & H. J. Deuticke**
1934. Zeit. physiol. Chem. **230**: 50.

16. **Fischer, H. O. L., & E. Baer**
1932. Ber. Chem. Ges. **65**: 337.

17. **Meyerhof, O., & W. Kiessling**
1936. Biochem. Zeit. **283**: 83.

18. **Harden, A.**
1932. Alcoholic Fermentation. Longmans, Green & Co. London.

19. **Lundsgaard, E.**
1930. Biochem. Zeit. **217**: 162; **227**: 51.

20. **Dische, E.**
1934. Naturwiss. **22**: 855.

21. **Green, D. E., D. M. Needham, & J. G. Dewan**
1937. Biochem. Jour. **31**: 2327.

22. **Meyerhof, O., P. Ohlmeyer, & W. Möhle**
1938. Biochem. Zeit. **297**: 113.

23. **Warburg, O., & W. Christian**
1939. Biochem. Zeit. **303**: 40.

24. **Negelein, E., & H. Brömel**
1939. Biochem. Zeit. **303**: 132.

25. **Lipmann, F.**
1940. Jour. Biol. Chem. **134**: 463.

26. **Lipmann, F.**
1942. Federation Proc. **1**: 122.

27. **Bücher, Th.**
1942. Naturwiss, **30**: 756.

28. **Lehmann, H.**
1936. Biochem. Zeit. **286**: 336.

29. **Lewis, G. N.**
1923. Valence and the Structure of Atoms and Molecules. McGraw-Hill Book Co., Inc. New York.

30. **Pauling, L.**
1939. The Nature of the Chemical Bond. Cornell University Press, Ithaca. New York.

31. **Ingold, C. K.**
1939. Jour. Chem. Soc. 1120.

32. **Michaelis, L.**
1940. Ann. N. Y. Acad. Sci. **40:** 39.

33. **Kalckar, H. M.**
1941. Chem. Rev. **28:** 71.

34. **Meyerhof, O., & J. Suranyi**
1927. Biochem. Zeit. **191:** 106.

35. **Kalckar, H. M.**
1943. Jour. Biol. Chem. **148:** 127.

36. **Ochoa, S.**
1943. Jour. Biol. Chem. **149:** 577.

37. **Colowick, S. P., M. S. Welch, & C. F. Cori**
1940. Jour. Biol. Chem. **133:** 359.

38. **Kalckar, H. M.**
1939. Biochem. Jour. **33:** 631.

39. **Ochoa, S.**
1941. Jour. Biol. Chem. **138:** 751.

40. **Belitzer, V. A., & E. T. Tsibakova**
1939. Biokhimiya. **4:** 518.

41. **Meyerhof, O., & K. Lohmann**
1934. Biochem. Zeit. **273:** 60.

42. **Meyerhof, O., & W. Schulz**
1935. Biochem. Zeit. **281:** 292.

43. **Cori, C. F., & G. T. Cori**
1936. Proc. Soc. Exp. Biol. Med. **34:** 702.

44. **Cori, C. F., S. P. Colowick, & G. T. Cori**
1937. Jour. Biol. Chem. **121:** 465.

45. **Cori, C. F., G. T. Cori, & G. Schmidt**
1939. Science. **89:** 464.

46. **Kiessling, W.**
1939. Biochem. Zeit. **302:** 50.

47. **Cori, C. F., G. T. Cori, & A. A. Green**
1943. Jour. Biol. Chem. **151:** 39.

48. **Doudoroff, M.**
1943. Jour. Biol. Chem. **151:** 351.

49. **Baer, E.**
1942. Jour. Biol. Chem. **146:** 391.

50. **Annau, E.**
1934. Zeit. physiol. Chem. **224:** 141.

51. **Örström, Å.**
1937. Naturwiss. **25:** 300.

52. **Warburg, O., & W. Christian**
1936. Biochem. Zeit. **287:** 440.

53. **Hehre, E. J., & J. Y. Sugg**
1942. Jour. Exp. Med. **75:** 339.

54. **Binkley, F.**
1943. Jour. Biol. Chem. **150:** 261.

55. **Smythe, C. V.**
1944. Ann. N. Y. Acad. Sci.

THE SIGNIFICANCE OF COUPLED REACTIONS FOR THE ENZYMATIC HYDROLYSIS AND SYNTHESIS OF PROTEINS

By

Max Bergmann and Joseph S. Fruton
Rockefeller Institute for Medical Research, New York, N. Y.

Of the many problems which have excited the curiosity of the biochemist, there are few more challenging than that presented by the mechanism of the synthesis of proteins in living cells The biochemical journals of the past two decades are replete with discussions of the probable reaction paths followed during the biosynthesis of proteins. In reviewing the salient literature concerning this question, it seems best to start with the generally accepted view that proteins consist largely of amino acid residues that are linked to each other by means of peptide bonds. This theory is based on the experimental finding that when proteins are hydrolyzed by proteolytic enzymes or by acids or bases, there are formed amino acids and amino and carboxyl groups are liberated. It is natural, therefore, that the question has been asked whether the biosynthesis of a protein molecule involves a reversion of the hydrolytic action of the proteolytic enzymes. In other words, does protein synthesis begin with the condensation of a number of amino acids to form peptide bonds?

The idea that the hydrolytic action of proteolytic enzymes might be reversed under certain experimental conditions is not a new one. Numerous investigators in the late nineteenth and early twentieth century observed that when proteolytic enzymes were added to concentrated solutions of the products of proteolysis, precipitates were frequently observed.[1] Later workers noted a decrease in amino nitrogen in the reaction mixture.[2] These results were taken to indicate that protein synthesis had occurred, although the evidence presented for the protein nature of the precipitates was unconvincing. The further study of this problem was energetically pursued by Wasteneys and Borsook in the 1920's. These investigators showed that when pepsin is allowed to act on egg albumin at pH 1.6 and the solution of the products of peptic hydrolysis is adjusted to pH 4 and concentrated, addition of pepsin results in the formation of a precipitate, designated plastein.[3] This product was described as a protein largely on the basis of its digestibility by pepsin and its precipitation by trichloracetic acid. There has been considerable discussion in the literature concerning the protein nature of plastein. It may be sufficient to point out that the insolubility of plastein or its precipitability by trichloracetic acid is not evidence of its protein nature; neither is the fact that it is digested by pepsin at pH 1.7. From the recent work of Flosdorf[4] and others it seems probable that plastein represents a complex mixture of peptides of unknown chemical structure and that the average size of the individual peptides is rather small.

Of greater importance to this discussion is the question as to whether experiments performed under such complex conditions provide any evidence regarding the reversal of the hydrolytic action of proteolytic enzymes. In the first place, the formation of plastein gives no clear evidence of the formation of peptide bonds. Furthermore, the pH optimum for the so-called synthesis is quite different from that for the hydrolysis of proteins. In the absence of any evidence on this point, one may ask, therefore, whether there is any reason to believe that in the crude pepsin employed in the plastein experiments, there are different enzymes acting optimally on a given protein at various pH values. In that case, the synthesis of plastein might be effected at pH 4 by an enzyme or enzymes different from those that act hydrolytically at pH 1.6.

In discussing the theoretical aspects of plastein formation, it has been assumed, on the basis of the mass action principle, that one could apply the equilibrium equation

$$\frac{(\text{Products})^n}{\text{Protein}} = K$$

where n is the number of molecules of the hydrolysis products formed from a protein. On this assumption, increase of the concentration of the products should presumably favor synthesis. However, this picture is somewhat too simple because, as we shall see later, in the course of hydrolysis or synthesis in protein digests, a very complex sequence of reactions probably takes place in which the products of one reaction are in turn employed for a subsequent reaction. In the presence of a mixture of enzymes, some of which may not be proteolytic enzymes, some of these reactions may not involve the synthesis of peptide bonds at all. Such a complex situation scarcely warrants the introduction of the term "equilibrium constant." These remarks regarding the enzymatic formation of plastein do not dispute the idea that proteolytic enzymes can catalyze peptide synthesis; they are intended to indicate some of the uncertainties that arise when one attempts to study the question of protein synthesis with substrates of unknown chemical nature.

At the present stage of development of our experimental methods, clearer information is gained by limiting the study of peptide synthesis to chemical systems much simpler than those encountered in concentrated protein digests. This means, in the first place, that it is necessary to prove unequivocally that proteolytic enzymes can link two amino acids together to form a peptide bond. This has been accomplished by selecting suitable amino acid derivatives and by the isolation and identification of the peptide synthesized by the enzyme. Through this technique it has been possible to effect the synthesis of single CO-NH linkages by each of the following enzymes: papain, bromelin, intracellular proteolytic enzymes of liver and spleen, and chymotrypsin.[5, 6] Some of these enzymatic syntheses are presented in TABLE 1. These experiments give good evidence for the idea that proteolytic enzymes catalyze the attainment of equilibrium between a peptide and its split products and that, under suitable conditions, the enzymes can catalyze peptide synthesis.

TABLE 1

SYNTHESES BY PROTEOLYTIC ENZYMES

Enzyme: Papain

Benzoyl leucine + leucine anilide → Benzoyl leucyl leucine anilide

Benzoyl phenylalanine + leucine anilide → Benzoyl phenylalanyl leucine anilide

Carbobenzoxy phenylalanyl glycine + tyrosine amide →
Carbobenzoxy phenylalanyl glycyl tyrosine amide

Enzyme: Chymotrypsin

Benzoyl tyrosine + leucine anilide → Benzoyl tyrosyl leucine anilide

Benzoyl tyrosine + glycine anilide → Benzoyl tyrosyl glycine anilide

Well-defined reactions of this type provide an opportunity of studying the enzymatic synthesis of peptide linkages with respect to the specificity of the enzyme action, the effect of pH and enzyme activators, and the manner in which peptide synthesis might be coupled with other reactions. We should like to outline some of the results to date and to speculate concerning the possible future directions of such investigations.

First, with respect to the specificity of peptide synthesis. If we assume that the action of a proteolytic enzyme is to catalyze the attainment of equilibrium between a peptide and its hydrolytic products, then one should expect that the specificity of synthesis should be the same as that of hydrolysis. In other words, if we alter the chemical nature of one of the groups near a peptide bond in such a way that a given enzyme no longer is able to hydrolyze the bond, then a similar structural change in the components that are employed for the enzymatic synthesis will also prevent the formation of that peptide bond. For example, chymotrypsin requires for its hydrolytic action the presence, in its substrate, of the aromatic side chain of l-tyrosine or l-phenylalanine in the vicinity of the sensitive peptide bond. Replacement of this side chain with that of another amino acid such as leucine prevents hydrolysis.[7] It even suffices to replace the l-tyrosine residue in benzoyl-l-tyrosylglycine amide by a d-tyrosine residue to render the resulting benzoyl-d-tyrosylglycine amide resistant to chymotrypsin action. Although the experimental material on the specificity of peptide synthesis is less extensive than that available for peptide hydrolysis, there is every reason to believe that the same precise specificity which applies to the hydrolytic action of chymotrypsin as well as other proteolytic enzymes also applies to their synthetic action.

Whatever the specificity of synthesis or hydrolysis of peptide bonds by a given proteinase may be, the reaction naturally cannot manifest itself if the thermodynamic prerequisites for its occurrence are not fulfilled. The hydrolysis of peptide linkages in proteins, as well as in simple peptides, proceeds spontaneously in the presence of a suitable enzyme. This indicates that the equilibrium which is established between hydrolysis and synthesis (of a peptide bond) is almost always very far on the side of hydrolysis. One must conclude that the hydrolysis of peptide linkages is a reaction that involves a decrease in the total free energy. An approximate idea of the magnitude of the free energy change involved in this hydrolytic reaction may be gleaned from the thermal data of Borsook and Huffman.[8] According to their calculation, the reaction:

dl-leucylglycine (solid) + H_2O (liq.) → dl-leucine (sol.) + glycine (sol.),

will result in the liberation of approximately 3000 calories. It is also stated that determinations of thermal data on other peptides indicate that the free energy changes in the hydrolysis of other peptide bonds are of a similar order of magnitude.

From these considerations it follows that the process of peptide synthesis in homogeneous solution requires, for its occurrence, energy which must be supplied by another reaction. Thermodynamic data alone cannot tell us anything except the approximate amount of energy which will have to be introduced in order to make the total free energy change in the system negative. We must therefore look for the specific physical or chemical mechanisms that make possible the synthesis of peptide bonds.

Let us take the case of the peptide syntheses in the model experiments presented in TABLE 1. These syntheses occur because the products in all cases are rather sparingly soluble compounds. In the enzyme catalyzed reaction, therefore, the solution becomes super-saturated with respect to the synthetic product which then crystallizes from the solution. This crystallization disturbs the equilibrium and consequently more of the synthetic product is formed. The reaction thus proceeds in the direction of synthesis until the equilibrium ratio of concentrations has been established. In these peptide syntheses, therefore, the energy required for peptide synthesis comes from the process of crystallization whereby the synthetic product is removed from the equilibrium.

For example, benzoyl-tyrosyl-glycine-amide is specifically split by chymotrypsin to form benzoyl-tyrosine and glycine-amide. The hydrolysis is nearly 100 per cent complete if allowed to proceed for a sufficient length of time. If we try to reverse the hydrolytic process by allowing chymotrypsin to act on benzoyl-tyrosine and glycine-amide, we find that no appreciable synthesis has occurred. If we take glycine-anilide in place of glycine-amide, synthesis occurs and the expected product benzoyl-tyrosyl-glycine-anilide crystallizes out of the solution. The difference in the results with glycine-amide and glycine-anilide depends on the fact that the benzoyl-tyrosyl-glycine-amide is considerably more soluble than is the anilide. Under the experimental conditions of the enzyme experiment, the concentration of the amide at equilibrium is apparently much lower than that of a saturated solution of this compound. On the other hand, a saturated solution of the anilide is always at a lower concentration than the equilibrium concentration and as soon as a slight amount of the synthetic product is formed it is removed from the solution of crystallization.

Although the compounds employed in this model experiment are somewhat unphysiological, the mechanism which provides the energy for the peptide synthesis is not as unphysiological as might appear at first glance. We know that certain proteins are rather insoluble: collagen and elastin are two examples. It does not seem impossible that, in such cases, the energy for peptide synthesis may be provided, at least in part, by the removal of an insoluble synthetic product from the equilibrium. In general, we can say that any polyphase system which involves the removal of the synthetic product from the enzymatic equilibrium will favor peptide synthesis.

Another mechanism for favoring the formation of peptide bonds is to couple the synthetic reaction with another energy-yielding chemical reaction which removes the synthesized peptide from the equilibrium with its precursors. An example of such a coupled reaction is provided by the following model experiment:[9] The compounds, acetyl-dl-phenylalanyl-glycine and glycyl-l-leucine, when added separately to the proteolytic enzymes in papain, are not affected by the enzymes. However, when these two peptides are mixed and added to papain in aqueous solution, the glycyl leucine is hydrolyzed. This hydrolysis is accomplished through a series of three reactions. The first step involves a synthetic reaction in which the two peptides are joined to form acetyl-phenylalanyl-glycyl-glycyl-leucine. The driving force for this synthesis is provided by the subsequent enzymatic hydrolysis of the acetyl tetrapeptide. First a leucine residue is split off and then a glycine residue is removed. The compound acetyl-phenylalanyl-glycine, which is unchanged by this process, is termed a co-substrate.

It has been pointed out by Petrie,[10] with some justification, that this is no way to synthesize proteins, since the final result of our efforts in this model experiment is a hydrolysis rather than a synthesis. Nevertheless, this model experiment serves as additional evidence for the conclusion that proteolytic enzymes are capable of catalyzing the synthesis of peptide linkages if the synthesis is coupled to another energy-yielding reaction.

There is another aspect of this coupled synthesis and hydrolysis which deserves attention. In the experiment discussed above, two peptides are combined by an enzyme with the subsequent hydrolysis of two peptide linkages. Under physiological conditions, proteolytic enzymes will frequently have at their disposal a number of peptides, as, for example, in the course of the enzymatic hydrolysis of a protein. In such cases, we must now face the situation that a complex series of coupled reactions may ensue in which there occur both synthesis and hydrolysis of peptide bonds. The course of this sequence of coupled reactions will depend on the specificities of the enzymes that are involved and the concentration and the chemical constitution of the peptides that are present.

It follows, therefore, that the digestion of a protein by a mixture of proteolytic enzymes can no longer be visualized solely as a sequence of hydrolytic reactions. We must also take into account the possible occurrence of coupled reactions which involve synthetic processes as well.

A further consequence of the concept of co-substrate action arises when we consider the effect of the introduction of a foreign peptide or protein into a system containing several enzymes and peptides. If this foreign substance participates in the sequence of coupled reactions, its presence may actually alter the fate of the proteolytic system. Attention has been drawn to the possible significance of such co-substrate action in the biological action of foreign proteins, such as the viruses.[11]

All that has gone before has concerned the experimentally established mechanisms which can provide the driving force for the reversal of the hydrolytic action of the proteolytic enzymes. We are not in a position to state that reversal of proteolysis is actually the process employed by biological systems for the synthesis of peptide bonds. However, we know of no other enzymatic processes that show the precise specificity that characterizes the catalytic action of the proteolytic enzymes. At the present stage of knowledge concerning the biosynthesis of proteins, perhaps the best one can do is to continue to gather experimental information regarding the various types of energetic coupling which will result in peptide synthesis in well-defined chemical reactions catalyzed by proteolytic enzymes. We are certainly unable to state which of the many conceivable coupled reactions might actually occur in biological systems. In view of the multiplicity of proteins and the almost infinite possibilities of variation of their chemical structure, it would be surprising if there were only one or even only a few energy-yielding mechanisms involved in protein synthesis.

Several energy-yielding mechanisms may be envisaged. For example, in the case of the more complex proteins, such as the nucleoproteins, the chromoproteins, or the phosphoproteins, part of the driving force for synthesis of peptide bonds may be provided by chemical reactions involved in the linkage of the non-protein moiety to the amino-acid complex. We know from the extensive data now available on the specificity of proteolytic enzymes that, frequently, minor changes in the side chains of a peptide will decisively alter the sensitivity of the peptide to enzyme action.[5] Thus, benzoyl-lysine-amide is split by crystalline trypsin (FIGURE 1). If the epsilon amino group is acylated, however, the peptide bond becomes resistant to proteolytic action. Another case is that found by Posternak[12] who showed that a phosphorylated dipeptide isolated on partial degradation of casein, phosphoserylglutamic acid, is resistant to the action of the proteolytic enzymes of intestinal mucosa; however, when the phosphoric acid residue was split off by the action of phosphatase, the resulting phosphorus-free peptide was readily split. On the basis of what we already know, it seems reasonable to infer that a similar effect would be noted for the synthesis of the peptide. One may ask, therefore, whether in the course of the biosynthesis of a casein molecule, part of the energy for the synthesis of peptide bonds involving serine residues will be provided through coupling with phosphorylation reactions. Since methods are now available for the synthesis of peptides of l-serine,[13] this possibility can be explored experimentally. Previous papers presented at this conference have discussed the role of phosphate in enzymatic syntheses. The desirability of further experiments on the relation of phosphorylation to peptide synthesis therefore needs no additional emphasis.

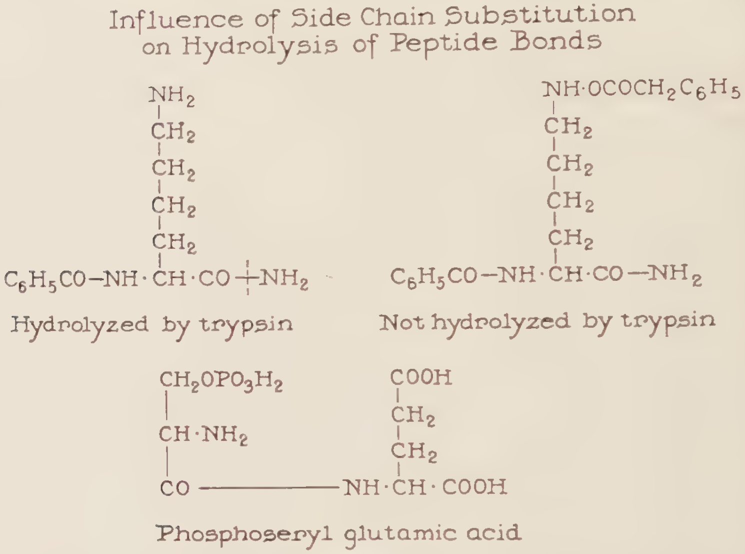


FIGURE 1.

The epsilon amino group of lysine and the β-hydroxyl group of serine are only two of the many reactive groups in the side chains of naturally occurring peptides and proteins. If one considers the possibilities of reaction with the imidazole group of histidine, the sulfhydryl group of cysteine, or other reactive amino acid side chains, the possible variation in the nature of the energy-yielding chemical reactions involving the side chains of peptides becomes considerable indeed.

To these structural changes in peptides which may favor removal of the synthetic product from the equilibrium, there must be added the possible formation of intramolecular links between different parts of the peptide chain. In the specific case of keratin, which is resistant to the action of proteolytic enzymes, Goddard and Michaelis[14] have shown that this protein, on treatment with sulfhydryl compounds, is transformed into a product which is easily hydrolyzed by trypsin and pepsin. This change in behavior toward proteolytic enzymes has been attributed to the rupture of disulfide linkages in keratin. In addition to such disulfide linkages, other bonds have been suggested which might fix the peptide chains of proteins in certain spatial arrangements and thus cause these chains to undergo folding in a rather specific manner. However, the nature of the linkages that are involved in such folding of the peptide chain is not known, although this question has been the subject of extensive and stimulating speculation.

Another possible source of energy for peptide synthesis is, of course, the coupling with oxidation-reduction reactions. Although the view has been expressed frequently that such coupling exists, and it seems reasonable that it must be so, there is still no clear-cut experimental evidence on the subject. Maver and Voegtlin[15] studied the effect of oxygen tension on the proteolysis of fibrin by papain. They concluded that proteolysis may be favored by the presence of sulfhydryl groups which on oxygenation may be converted into disulfide groups. It was assumed that the presence of these disulfide compounds favored protein synthesis. The difficulties that arise in the interpretation of these results are similar to those encountered in the discussion of the plastein formation in concentrated peptic digests. The complex mixture of peptides of unknown composition present in these oxygenation experiments makes it almost impossible to determine definitely the nature of the chemical reactions that are going on. Actually, as Linderstrom-Lang[16] and Harris[17] have shown, on oxygenation there may occur the formation of intermolecular disulfide linkages between peptides, thus giving rise to substances more insoluble than the parent substances. Mothes[18] has also claimed a dependence of protein synthesis on oxygen supply, in this case for plants; however, here also the situation is unsettled and much further work is needed.

In the interpretation of these experiments of Voegtlin and Mothes, emphasis was placed on the essential role of sulfhydryl compounds in the activation of intracellular proteolytic enzymes. It was implied that by converting the sulfhydryl groups of a proteolytic enzyme into disulfide groups, the enzyme would cause peptide synthesis instead of hydrolysis. When this idea was tested in experiments with simple peptides of known structure, it was found that the same activation requirements were exhibited in the synthetic reaction as were found for the hydrolytic reaction.[5] Whenever an intracellular enzyme required activation by HCN or cysteine for hydrolysis, similar activation was found to be necessary for synthesis. If we view the activation process as the conversion of an inactive precursor into an active enzyme, then it would not be understandable from a thermodynamic point of view how the same enzyme could cause an equilibrium reaction to proceed in opposite directions depending solely on the state of activation of the enzyme.

Our knowledge of the mechanism of the activation of the intracellular proteolytic enzymes by HCN or sulfhydryl compounds is still incomplete. This is due, in large part, to the fact that they have not been purified to the same extent as have the enzymes of the digestive tract. At the present time, the experimental facts are best explained by the following picture of the activation process.[19] An inactive **α**-proenzyme is converted by traces of reducing substances into a second β-form that is also proteolytically inactive (FIGURE 2). However, the second form

ACTIVATION OF INTRACELLULAR PROTEINASES

$$\underset{\text{(inactive)}}{\alpha\text{-proenzyme}} \underset{\text{oxidation}}{\overset{\text{reduction}}{\rightleftharpoons}} \underset{\text{(inactive)}}{\beta\text{-proenzyme}}$$

$$\begin{tikzcd} \underset{\text{(inactive)}}{\beta\text{-proenzyme}} + \text{activator} \arrow[d, shift left] \\ \underset{\text{(active)}}{\text{activator-enzyme compound}} \arrow[u, shift left] \end{tikzcd}$$

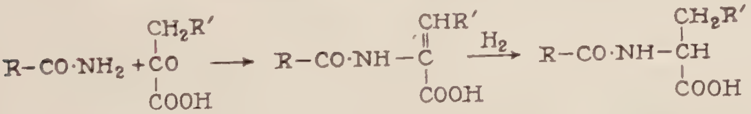
FIGURE 2.

differs from the first in that it can combine with substances such as HCN, H_2S, cysteine or glutathione, to become an active proteolytic enzyme. The formation of the active enzyme requires relatively large concentrations of the activator. Not all compounds that are capable of forming addition compounds with the β-proenzyme can also convert the **α**-form to the β-form. An example of HCN which, even in high concentrations, cannot reduce the **α**-form to the β-form. If a trace of cysteine is added, however, the β-proenzyme is formed which, in turn, can combine with HCN to form an active enzyme. Of particular interest in this scheme is the fact that the enzyme-activator compounds that are formed are readily dissociable. If a volatile activator such as HCN or H_2S is employed, it is possible to inactivate the enzyme by simply removing the activator under reduced pressure.

It does not seem likely, however, that the intervention of oxidation-reduction reactions in the conversion of the α-proenzyme to the β-proenzyme will provide the requisite driving force of peptide synthesis by proteolytic enzymes. Thus far, no information is available concerning the precise nature of the biological oxidation-reduction systems that may be coupled with protein synthesis. Certainly, this problem presents one of the most interesting directions for future research.

Thus far, we have discussed the problem of the synthesis of peptide bonds in terms of the energetic coupling required to reverse the hydrolytic action of the proteolytic enzymes. As was indicated before, perhaps the strongest reason for believing that the enzyme catalyzed condensation of amino acids represents the most probable metabolic course of protein synthesis is the fact that the proteolytic enzymes, by virtue of their sharp specificity, are the only known biocatalysts which could direct, precisely and reproducibly, the complex sequence of successive peptide syntheses required for the formation of a protein. An additional reason for giving the proteolytic enzymes serious consideration in the scheme of protein synthesis emerges from the researches of Schoenheimer on the metabolism of isotopic amino acids. His work led him to conclude that, in the dynamic equilibrium between proteins and amino acids in the tissues, peptide bonds are constantly being broken and reformed under the catalytic influence of the tissue enzymes.[20]

Nevertheless, in view of the present state of our knowledge, we cannot overlook the possible biological formation of peptide bonds by mechanisms other than the reversal of proteolysis. For example, it is known that acid amides can react with keto acids according to the following sequence of reactions to yield substituted dehydro amino acids.[21]



$$\mathrm{R{-}CO{\cdot}NH_2} + \underset{\displaystyle \mathrm{COOH}}{\overset{\displaystyle \mathrm{CH_2R'}}{\mathrm{CO}}} \longrightarrow \mathrm{R{-}CO{\cdot}NH{-}}\underset{\displaystyle \mathrm{COOH}}{\overset{\displaystyle \mathrm{CHR'}}{\overset{\|}{\mathrm{C}}}} \xrightarrow{\mathrm{H_2}} \mathrm{R{-}CO{\cdot}NH{-}}\underset{\displaystyle \mathrm{COOH}}{\overset{\displaystyle \mathrm{CH_2R'}}{\mathrm{CH}}}$$

If an amino-acid amide would react in this manner a dehydropeptide would be formed. The coupling of this reaction with a reductive process would result in the formation of peptides. The existence of an enzyme apparatus required for such a series of reactions *in vivo* has not been demonstrated as yet, possibly because a systematic search has not been made. However, the biological occurrence of dehydropeptides is suggested by the presence, in animal tissues, of an enzyme system specifically adapted to the hydrolysis of dehydropeptides.[22] As a result of this enzymatic action, an amino acid, ammonia and a keto acid are formed. It seems of particular importance to investigate this enzyme reaction further and to determine whether, under suitable environmental conditions, it can be reversed. The recent developments in the synthetic methods for preparing dehydropeptides make available a large number of substrates for such enzyme experiments.[23]

To continue this line of thought a bit farther, the important role of glutamine in animal metabolism, recently demonstrated by Van Slyke, Archibald and Hamilton,[24] raises the question whether this acid amide can react with keto acids with the resulting synthesis of CO-NH bonds involving the γ-carboxyl of glutamic acid. Of course, glutathione is the best known naturally occurring substance that has such a bond.

Still another chemical mechanism for the formation of peptide bonds is provided by the series of reaction shown in FIGURE 3 in which a pyruvoyl amino acid is formed.[25] Herbst and Shemin[26] have recently converted pyruvoyl alanine to the oxime, which on catalytic reduction yielded alanylalanine. The dipeptide could also be prepared from the ketopeptide by catalytic hydrogenation in the presence of ammonia, according to the method of Knoop. A third method for converting the ketopeptide to the dipeptide has been suggested by Linderstrom-Lang[27] and Agren.[28] According to this scheme, the ketopeptide becomes aminated by a transamination reaction with glutamic acid. The complete Linderstrom-Lang scheme assumes the initial reaction of a ketoaldehyde such as methyl glyoxal with an amino acid, followed by an oxidation to the ketopeptide.

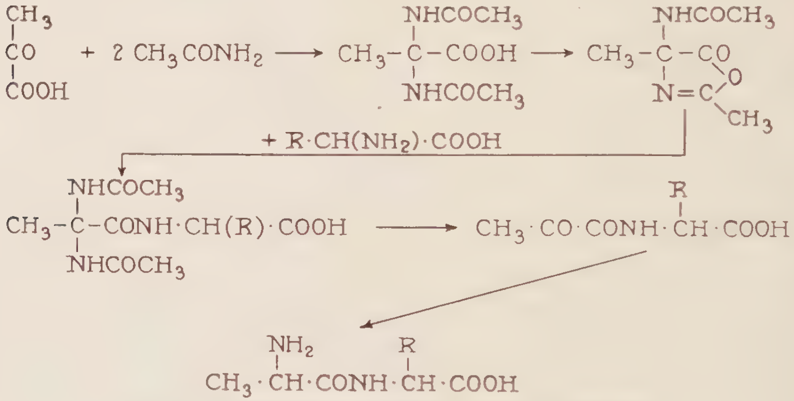


FIGURE 3. Formation of pyruvoyl amino acids and dipeptides.

These considerations concerning the possible chemical mechanisms for peptide synthesis are based partly on *in vitro* experiments and partly on speculation. The value of suitably chosen *in vitro* experiments for the study of chemical reactions *in vivo* is well illustrated by the history of our knowledge concerning the metabolic fate of arginine and its relation to creatine formation. In 1927 it was shown[29] that the series of chemical reactions shown in FIGURE 4 could be made to occur. In essence, they represent the transfer of an amidine group from arginine to glycine. A similar transfer was also effected *in vitro* from arginine to sarcosine leading to creatine. These experiments drew attention to the possible biological transfer of an amidine group from arginine to glycine. The recent experiments of Borsook[30] and Schoenheimer[31] have actually shown the biological occurrence of this process.

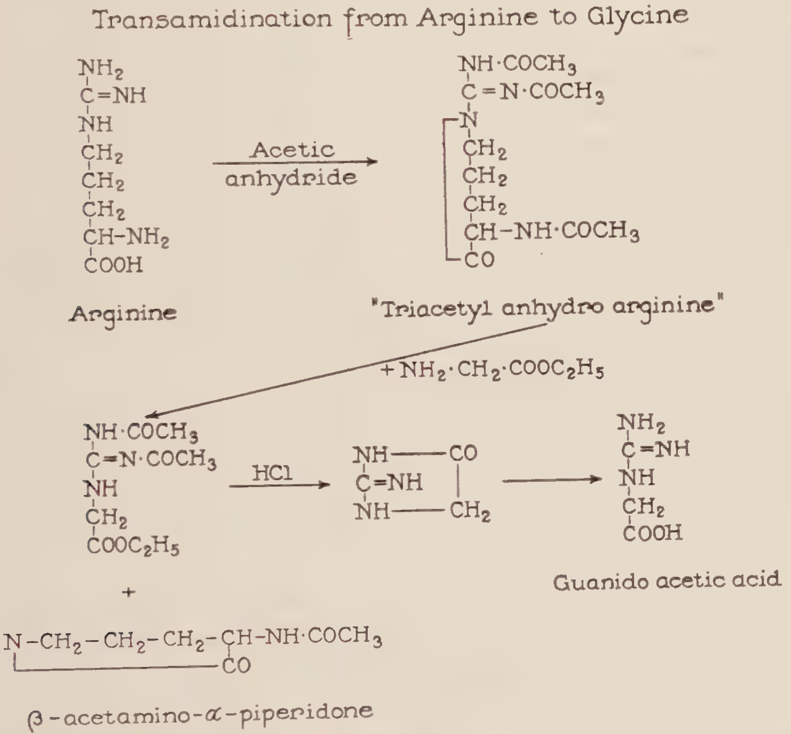


FIGURE 4.

From the experimental results and the speculative considerations which have been presented in this paper, it follows that future progress on the problem of the biosynthesis of proteins and peptides depends on a more intensive investigation of coupled reactions with well-defined enzyme systems and with substrates of known structure. Although the synthetic methods for preparing amino acid derivatives and peptides are far advanced, the purification of the tissue enzymes involved in peptide synthesis has lagged behind. We may be confident that, when the requisite intracellular enzymes are available in pure form, further progress in our knowledge of coupled proteolytic reactions will be forthcoming.

BIBLIOGRAPHY

1. **Sawjalow, W. W.**
   1901. Pflügers Arch. **75**: 171.
2. **Henriques, V., & I. K. Gjaldbäk**
   1912. Z. Physiol. Chem. **81**: 439.
3. **Wasteneys, H., & H. Borsook**
   1930. Physiol. Rev. **10**: 110.
4. **Flosdorf, E. W.**
   1941. Science **93**: 157.
5. **Bergmann, M., & H. Fraenkel-Conrat**
   1937. Jour. Biol. Chem. **119**: 707.
6. **Bergmann, M., & J. S. Fruton**
   1941. Advances in Enzymology **1**: 63.
7. **Bergmann, M., & J. S. Fruton**
   1937. Jour. Biol. Chem. **118**: 405.
8. **Borsook, H. & H. M. Huffman**
   1938. In C. L. A. Schmidt. Chemistry of amino acids and proteins. Baltimore. p. 865 (cf. also errata in Addendum, 1943).
9. **Behrens, O. K., & M. Bergmann**
   1939. Jour. Biol. Chem. **129**: 587.
10. **Petrie, A. H. K.**
    1943. Biol. Rev. **18**: 105.
11. **Bergmann, M.**
    1942. Advances in Enzymology **2**: 66.
12. **Posternak, T., & H. Pollaczek**
    1941. Helv. Chim. Acta **24**: 921.
13. **Fruton, J. S.**
    1942. Jour. Biol. Chem. **146**: 463.
14. **Goddard, D. R., & L. Michaelis**
    1934. Jour. Biol. Chem. **106**: 605.
15. **Maver, M. E., & C. Voegtlin**
    1939. Enzymologia **6**: 219.
16. **Linderstrom-Lang, K., & G. Johansen**
    1939. Enzymologia **7**: 239.
17. **Geiger, W. B., W. I. Patterson, C. R. Mizell, & M. Harris**
    1941. Jour. Res. Nat. Bur. Stand. **27**: 459.
18. **Mothes, K.**
    1933. Planta. **19**: 117.
19. **Irving, G. W. Jr., J. S. Fruton, & M. Bergmann**
    1941. Jour. Biol. Chem. **139**: 569.

20. **Schoenheimer, R.**
 1942. Dynamic state of body constituents. Cambridge, Mass.
21. **Bergmann, M., & K. Grafe**
 1930. Z. Physiol. Chem. **187**:187.
22. **Bergmann, M., & H. Schleich**
 1932. Z. Physiol. Chem. **205**: 65.
23. **Doherty, D. G., J. E. Tietzman, & M. Bergmann**
 1943. Jour. Biol. Chem. **147**: 617.
24. **Hamilton, P.**
 1942. Jour. Biol. Chem. **145**: 711.
25. **Bergmann, M., & K. Grafe**
 1930. Z. Physiol. Chem. **187**: 196.
26. **Shemin, D., & R. M. Herbst**
 1938. Jour. Am. Chem. Soc. **60**: 1951.
27. **Linderstrom-Lang, K.**
 1939. Ann. Rev. Biochem. **8**: 37.
28. **Agren, G.**
 1940. Acta Physiol. Skand. **1**: 233.
29. **Bergmann, M., & L. Zervas**
 1927. Z. Physiol. Chem. **172**: 277.
30. **Borsook, H., & J. W. Dubnoff**
 1941. Jour. Biol. Chem. **138**: 389.
31. **Bloch, K., & R. Schoenheimer**
 1941. Jour. Biol. Chem. **138**: 179.

SOME ENZYME REACTIONS OF SULFUR COMPOUNDS AND THEIR POSSIBLE INTERRELATIONSHIP

By C. V. Smythe
Rohm and Haas Company, Philadelphia, Pa.

Present information indicates that the three compounds, methionine, thiamine and biotin can supply the sulfur requirements of the mammalian organism under normal conditions. Methionine contains its sulfur as a thioether. Thiamine contains a thiazole ring and biotin contains a thiophene ring. The fact that these three compounds are required indicates that they are not interconvertible. Actually, a considerable amount of the sulfur requirement is supplied by the amino acid cysteine. It contains its sulfur in the sulfhydryl form. Thiamine may also exist to some extent in the sulfhydryl form. The thiazole ring opens rather easily under some conditions, forming a sulfhydryl group.[1, 2]

A striking property of the sulfhydryl groups is that they are easily oxidized with the formation of disulfides. The disulfide corresponding to the sulfhydryl form of thiamine has been isolated and shown to retain its vitamin activity.[3] The vitamin may actually function as a sulfhydryl compound. The disulfide of cysteine is, of course, the well-known cystine. All exclusively peptide compounds of cysteine retain a free sulfhydryl group, and they are free to undergo oxidation to the disulfide except in so far as steric factors prevent it. It is then a matter of some interest to know at what potential level the oxidation can occur for different sulfhydryl compounds. The potential level of the cysteine-cystine system has been the subject of considerable work. As is well known, cysteine and cystine, as we usually know them, do not behave in a thermodynamically reversible manner at a noble metal electrode. In many cases, the potential measured is independent of the cystine concentration.[4] Perhaps the most obvious explanation of the system is in terms of Dr. Michaelis' free radicals. According to it, cystine is not the primary oxidation product of cysteine but is a stabilization product of the free radical formed by the oxidation (i.e., it is the dimer of the free radical). Since the disulfide bond of cystine is relatively stable, the concentration of free radical existing in equilibrium with it is too small to enable it to establish a potential at an electrode.

I think one might expect, from such a view, that different cysteine-containing compounds might vary in the stability of their disulfide form and, hence, they might impart a quite different potential to an electrode and might tend more toward thermodynamic reversibility. Thus, a protein sulfhydryl system might be more reversible than the cysteine-cystine system. In this connection, a comparison of the cysteine and glutathione systems is interesting. Some of the electrometric measurements indicated that the potentials established were about the same for different sulfhydryl compounds.[5] The data of Fruton and Clarke[6] on measurements of equilibria with dyes also indicated about the same value for cysteine and glutathione although their value was lower than the electrometric measurements. Dr. Rieser and I recently had occasion to test the reduction of both disulfide compounds polarigraphically. Although the method is not as simple as we might wish, it showed a distinct difference between the two compounds. We did not get the same result on oxidizing the SH compounds, but this behavior might be expected from available information. In a solution of pH 2.3 the midpoints of the reduction waves differed by 140 mv., the cystine being the more negative. In view of this result, we were interested to learn of the work of Ryklan and Schmidt[7] at the University of California on the titration of these two compounds. They found that if cysteine was oxidized by iodine or iodate in the presence of excess iodide ion they got apparently normal titration curves. They got the same $E^{\circ\prime}$ values when cysteine and cystine were mixed in iodide containing solutions. Both methods showed an $E^{\circ\prime}$ for the system of + 0.27V. The $E^{\circ\prime}$ value was calculated according to the following equation:

$$E^{\circ\prime}_{(\mathrm{RSSR\text{-}RSH})\mathrm{pt}} = \mathrm{E\ (Obs)} + 0.283 - 0.059 \log \frac{(\mathrm{RSSR})^{\frac{1}{2}}}{(\mathrm{RSH})} + 0.059\ \mathrm{pH}.$$

Other comparable data for glutathione indicate an $E^{\circ\prime}$ of + 0.45V. If such differences are real, then it would seem likely that SH groups in proteins may vary significantly in their normal potentials. The problem might well repay additional work.

Mammalian tissues contain only very small amounts of free cysteine or cystine, but all of them contain appreciable amounts of glutathione. A peculiarity in the structure of glutathione is that the glutamic acid is linked by the γ-carboxyl group. As far as we know, the glutathione structure does not exist preformed in protein but must be formed by some special enzyme or in some special manner. Our attention was recently directed to this problem in the following way. Dr. Neubeck and I had occasion to determine the total reducing material present in guinea pig liver. To do so we deproteinized with metaphosphoric acid and titrated with iodine. We were surprised to find that the iodine titration increased rather rapidly as the time between the killing of the animal and the addition of metaphosphate increased. When this time was thirty minutes the titration was about 100 per cent greater than when the time was about two minutes. On checking into the matter, we found that much the same thing had been observed before and had been explained in different ways.[8, 9, 10, 11] We were struck by the fact that the character of the end point changed with the increase in titration. Originally, it was sharp and definite as the end point with glutathione is under such conditions. Later, it was much less sharp and definite and resembled that with cysteine. It seemed to us that the increased iodine titration might be best explained, as Bierich and Rosenbohm[11] had suggested, by a hydrolysis of glutathione and liberation of cysteine. The figures in TABLE 1 show how the iodine titration, under such conditions, changes when the total SH is kept constant, but the ratio of glutathione to cysteine changes.

TABLE 1

Ratio glutathione/cysteine	5:0	4:1	3:2	2:3	1:4	0:5
mls. I_2 reduced	5.65	7.41	9.65	11.62	13.43	14.03

TABLE 2

FREE CYSTINE AND IODINE TITRATION VALUES OF DEPROTEINIZED EXTRACTS OF GUINEA PIG LIVER

Time After Removal of Liver from Animal (Minutes)	0	10	15	20	30	40	50	60
Animal 1								
Iodine titration*	12.5	....	16.1	17.1	....	21.6	....	22.6
Colorimetric cystine†	0.19	....	0.40	0.58	....	0.89	....	1.00
Animal 2								
Iodine titration	13.3	15.2	....	19.4	....	24.1	....	25.4
Colorimetric cystine	0.18	0.36	....	0.71	....	0.98	....	1.08
Animal 3								
Iodine titration	13.1	15.4	....	21.3	....	26.3	....	26.3
Colorimetric cystine	0.17	0.35	....	0.74	....	0.90	....	0.87
Animal 4								
Iodine titration	7.0	....	....	8.5	10.0	10.5	11.3	11.4
Colorimetric cystine	0.16	....	....	0.53	0.68	0.78	0.80	0.80
Animal 5								
Iodine titration	14.6	17.9	....	20.6	23.1	25.0	....	25.6
Colorimetric cystine	0.31	0.56	....	0.75	0.93	0.93	....	0.87

* Expressed as milliliters of 0.001M iodine per gram of tissue.
† Expressed as milligrams of cystine per gram of tissue.

In keeping with such an explanation was the fact that determinations by the Sullivan method[12] on successive preparations showed a definite increase in cystine paralleling the iodine titration (TABLE 2). Also, in keeping with the explanation, were the facts that the nitrogen of the deproteinized extracts prepared at successive intervals and the total cystine, determined after hydrolysis, did not change during the time that the iodine titration changed (TABLE 3). When more glutathione was added an additional change in titration and in cysteine occurred. In such cases, it was possible to isolate the cystine in good yield. From such findings, we conclude that the glutathione was being hydrolyzed and in amounts sufficient to account for the increased iodine titration.

TABLE 3

FREE CYSTINE, TOTAL CYSTINE AND KJELDAHL NITROGEN IN DEPROTEINIZED EXTRACTS OF GUINEA PIG LIVER

Minutes of incubation before deproteinization	0	13	23	33	43
Colorimetric cystine mg. per gram tissue	0.25	0.38	0.64	0.75	0.83
Total cystine after hydrolysis mg. per gram tissue	0.94	0.81	0.81	0.88	0.83
Kjeldahl nitrogen mg. per gram tissue	1.22	1.05	1.07	1.07	. . .

From the fact that, in the liver fixed as rapidly as possible, the free cysteine is almost nil and the glutathione is quite appreciable (at least 2–3 mg. per gram), it is apparent that the equation representing the hydrolysis is pushed far to the glutathione side. In contrast, in the liver preparations, the glutathione is spontaneously hydrolyzed and the equilibrium reached corresponds to a large degree of hydrolysis. It seems to follow that, in the intact animal, the synthesis of liver glutathione must be coupled with an energy-yielding reaction.

It is quite likely that the enzyme concerned in this glutathione hydrolysis is the same as the one described originally as antiglyoxalase[13] and studied in more detail later.[14, 15] For some reason, guinea pig liver is rich in the enzyme and rat liver is poor in it. According to our results, it was rather difficult to extract the enzyme from the liver. Much of the activity remained in the normally insoluble portion, but a solution with some activity could be obtained. The enzyme was found to have a rather alkaline pH optimum of 8.5–9.0 (FIGURE 1). Dialysis caused a prompt inactivation. If the dialysate was concentrated and readded to the non-dialyzable material, the activity returned. Similarly, a heated extract reactivated the dialyzed material and, rather in-

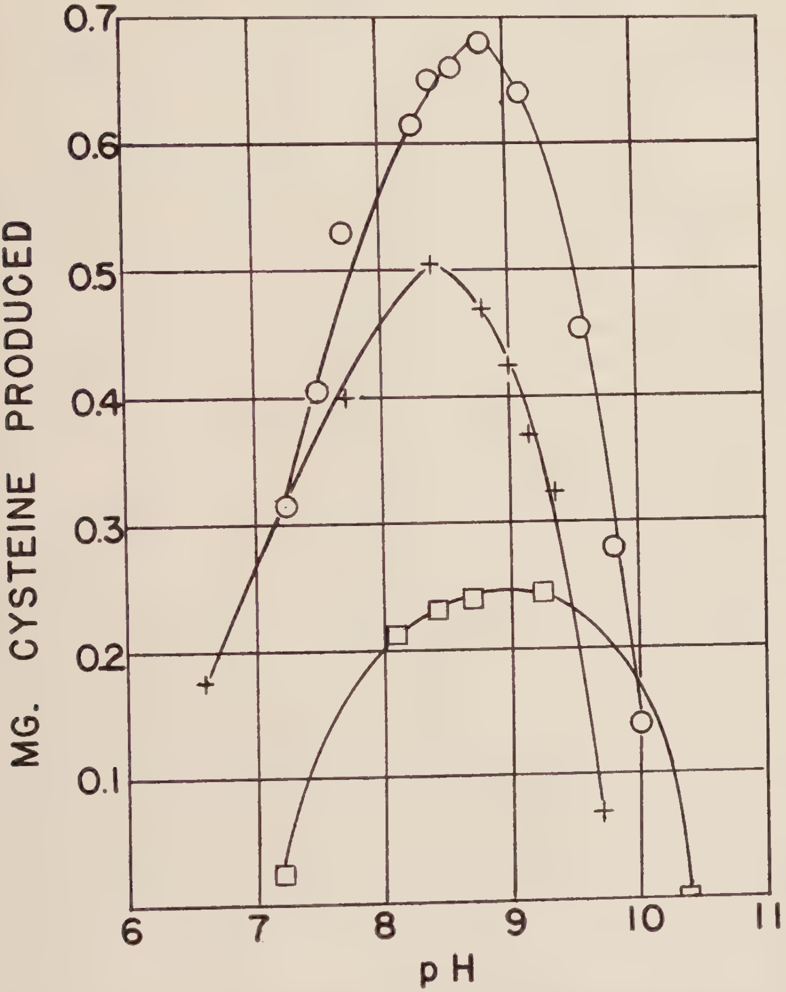


Figure 1. Optimum pH of Cysteine Production.

Production of cysteine by enzyme preparations as determined by the colorimetric method. The two upper curves indicate the production of cysteine by 2.0 ml. of guinea pig homogenate in 20 minutes at 25° in the presence of 2.0 mg. glutathione in a total volume of 2.5 ml. The pH of the mixture was obtained with glycine buffer (x) and acetate-veronal buffer (o). The lower curve indicates the production of cysteine by 2.0 ml. of partially purified enzyme preparation in 30 minutes, at 25° in the presence of 2.0 mg. glutathione, in a total volume of 2.5 ml. Acetate-veronal buffer was used to adjust the pH of the mixture.

terestingly, an extract of rat liver that had only slight activity by itself readily activated the dialyzed enzyme from guinea pig liver. Apparently, rat liver contains the coenzyme but not much of the enzyme. The coenzyme is relatively stable. Irradiation with a mercury lamp for 2.5 hours did not inactivate it. Heating for one hour at 100° C., in either 0.5 N acid or 1.0 N alkali, caused only slight destruction. Wet ashing caused complete destruction. All of these results are included in TABLE 4.

Table 4

Restoration of Activity to Dialyzed Enzyme Material

Material	Per Cent Activity
Original extract	100
" " dialyzed 20 hrs.	48
" " " 43 "	39
" " " 89 "	30
43 hr. dialyzed prep. + dialysate to orig. conc.	87
" " " + equal vol. heated extract	83
" " " + " " 1.5 rat liver homogenate	78
89 hr. " " + dialysate to orig. conc.	87
" " " + " irradiated 2.5 hrs. at pH 8.4	96
" " " + " heated 1 hr. at 100° C. in 0.5 m HCl	78
" " " + " heated 1 hr. at 100° C. 1.0 m NaOH	74
" " " + " ashed	30

It is obvious that the organism makes some effort to keep glutathione intact and one may wonder what advantages it offers over the constituent amino acids. Of course, it may play a role in the synthesis of protein, but not much data is available on that. Only one specific function is known—that of coglyoxalase—and there is some doubt about how important a role the glyoxalase enzyme system plays. As far as the reducing properties are concerned, free cysteine contains the same reducing group, although, as stated above, the potentials are not the same. Of course, an obvious difference is that oxidized glutathione is more soluble than cystine, but another possible advantage is that glutathione may be more stable than cysteine to the action of some other enzymes present in tissues.

An example of such an enzyme is the one that produces H_2S from cysteine and has no effect on glutathione. The presence of such an enzyme in mammalian tissues was first observed by Fromageot and his coworkers in 1939.[16] They observed the enzyme in dog liver and have since published several papers dealing with it. I first became acquainted with the enzyme largely by accident. We were studying the possible formation of ascorbic acid by rat tissues when it was observed.[17]

Fromageot and coworkers[18] came to the conclusion that the reaction involved was

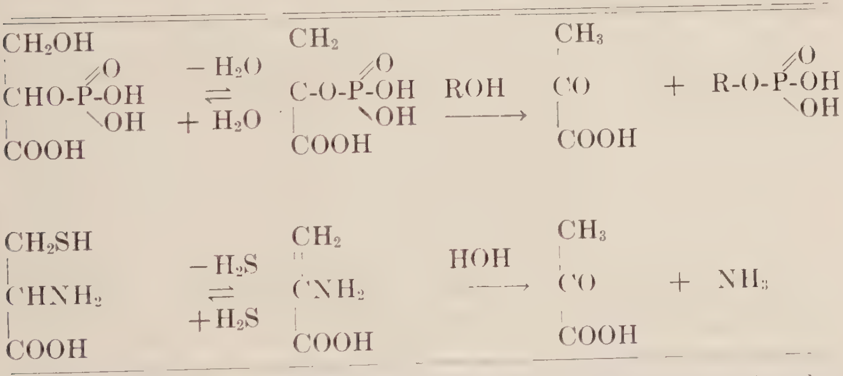
$$3\ \text{Cysteine} \rightleftharpoons \text{Cystine} + H_2S + \text{Alanine}.$$

They emphasized that the enzyme was quite different from the enzyme in some bacteria that produces both H_2S and ammonia from cysteine.[19] We were not able to confirm the above equation, but it may be correct to some extent. There does seem to be a tendency to form some cystine and small amounts of alanine. It might be possible to get conditions that would favor these products, but our results are much more in accord with the following equation[17]:

$$CH_2SHCHNH_2COOH \rightarrow CH_3COCOOH + H_2S + NH_3$$

We were interested to see whether or not this H_2S formation could be reversed, so we allowed the reaction to occur in the presence of added H_2S containing radioactive sulfur.[20] After part of the cysteine had been converted the reaction was stopped and the cysteine present was isolated. It was found to contain appreciable amounts of radioactive sulfur. We consider this proof that sulfide sulfur was converted to cysteine sulfur and, since we do not know any other method of accomplishing this conversion, we will assume that the reaction that produces H_2S was reversed. All of our attempts to start with pyruvic acid, ammonia and H_2S, and demonstrate a formation of cysteine, were unsuccessful. These findings naturally suggest a comparison with the formation of pyruvic acid from 1-phosphoglyceric acid (TABLE 5). In this case, the first reaction is the removal of H_2O with the formation of phosphopyruvic acid and this reaction is readily reversible. The next step is the removal of phosphoric acid by a phosphate acceptor or by H_2O with the production of pyruvic acid, and this step is not reversible. A difference in the two cases is that in the phosphoglycerate case, the intermediate compound, phosphopyruvic acid, is stable enough to be

TABLE 5



$$\begin{array}{l}CH_2OH\\ |\\ CHO\text{-}\overset{\displaystyle /\!\!/O}{P}\text{-}OH\\ |\qquad\quad \diagdown OH\\ COOH\end{array} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \begin{array}{l}CH_2\\ \|\\ C\text{-}O\text{-}\overset{\displaystyle /\!\!/O}{P}\text{-}OH\\ |\qquad\quad \diagdown OH\\ COOH\end{array} \xrightarrow{ROH} \begin{array}{l}CH_3\\ |\\ CO\\ |\\ COOH\end{array} + R\text{-}O\text{-}\overset{\displaystyle /\!\!/O}{P}\text{-}OH \atop \qquad\qquad \diagdown OH$$

$$\begin{array}{l}CH_2SH\\ |\\ CHNH_2\\ |\\ COOH\end{array} \underset{+H_2S}{\overset{-H_2S}{\rightleftharpoons}} \begin{array}{l}CH_2\\ \|\\ CNH_2\\ |\\ COOH\end{array} \xrightarrow{HOH} \begin{array}{l}CH_3\\ |\\ CO\\ |\\ COOH\end{array} + NH_3$$

worked with, while in the cysteine case, the supposed intermediate, amino acrylic acid, is not stable. According to Chargaff and Sprinson[21] and Binkley,[22] serine undergoes a similar change and is converted to pyruvic acid via amino acrylic acid. Thus, pyruvic acid is produced enzymatically from three relatively different compounds by what appear to be quite similar reactions. Such findings are in very good agreement with the earlier work of Bergmann and coworkers[23] on the chemical behavior of such compounds. It has been suggested that the same enzyme may be acting on all three compounds.[21, 22]

Some information is available on the partial purification and properties of the H_2S forming enzyme. It is inhibited by cyanide[16, 17] by As_2O_3[25] and by such carbonyl reagents as phenylhydrazine, hydroxylamine and semicarbazide.[25] The activity of the crude tissue preparations is not inhibited by NaF,[16, 17] but the purified enzyme is inhibited.[22] Some other amino acids do not inhibit, but other thiol compounds do.[25] Magnesium apparently forms a component part of the enzyme[25, 22, 21] although it can be replaced by some other metals.[22]

The question of a name for the H_2S forming enzyme is of some interest. Fromageot and coworkers called the bacterial enzyme that produces H_2S and NH_3 from cysteine, cysteinase; and they called the one in mammalian tissue that, according to them, produces H_2S but no NH_3, desulfurase. It is doubtful if these enzymes differ, at least in the manner suggested, and hence two names are possibly too many. The term cysteinase is not very descriptive. The term cysteine desulfurase probably causes no confusion at the present time, but if the suggested mechanism of the reaction is correct the name would seem to be incorrect. It is at least theoretically possible to remove sulfur, as such, from cysteine and an enzyme that could do so would properly be a desulfurase. However, the fact that hydrogen is removed along with the sulfur is rather an important one, and, I think, should be indicated in the name if possible. Calling an enzyme that removes H_2S a desulfurase is analogous to calling an enzyme that removes H_2O a deoxygenase. If we call the addition of H_2O a hydration and an enzyme that removes H_2O a dehydrase then it would seem reasonable to call the addition of H_2S a sulfhydration, and an enzyme that removes H_2S desulfhydrase. The enzyme that produces H_2S from cysteine would then be cysteine desulfhydrase.

In view of the fact that H_2S is very toxic for an intact animal, it seemed curious that tissues should contain an enzyme for producing it. The possible connection of such H_2S formation with the known toxicity of cysteine for young white rats under certain conditions seemed obvious. Actually, we could find no data on the effect of H_2S on isolated tissues, so we tested the effect of adding H_2S to liver, kidney and brain preparations.[26] It turned out that the oxygen consumption of liver and kidney was not at all sensitive to H_2S, while that of brain was quite sensitive. Apparently the toxicity of H_2S is not a general tissue phenomenon. Such a finding could be utilized in studying the enzyme reactions involved.

The fact that methionine is an essential amino acid and cannot be replaced by cystine is well established.[27] The fact that its methyl group can be removed and transferred to other compounds is also well established.[28] Apparently, the transmethylation is reversible, for methyl groups supplied as choline can be recovered in methionine.[29] The enzymes concerned in this reaction have not been studied in detail, as far as I know. The homocysteine resulting from the demethylation of methionine is another sulfhydryl compound. The addition of it to liver preparations results in the formation of some hydrogen sulfide.[17, 30] According to my measurements, much less H_2S is produced from homocysteine than from cysteine, and I would normally assume that both compounds are probably acted upon by the same enzyme. However, Fromageot and Desnuelle[31] report that the ratio of the activity on the two compounds is quite different for different enzyme preparations and they conclude that they are acted upon by different enzymes. The other products resulting from this action have not been determined.

Binkley and du Vigneaud[32] have found that homocysteine plus serine, in the presence of liver extracts, produces cysteine. It is interesting that in this case also pyruvic acid plus ammonia will not replace serine. With my extracts, I was not able to replace homocysteine with H_2S. Binkley and du Vigneaud did not obtain cysteine when they used methionine instead of homocysteine, but Floyd and Medes[33] report that methionine added to liver or kidney slices, under aerobic conditions, results in cysteine formation. However, the amount of cysteine obtained was very small.

The sulfhydryl group can be oxidized further than the disulfide stage and can produce inorganic sulfate, or, in the case of cysteine, cysteic acid. Some tissues contain an enzyme that decarboxylates cysteic acid,[34, 35] producing taurine. Thus, an enzymatic path is charted for the conversion of methionine to cysteine and to taurine, reactions that were well established on intact animals.

BIBLIOGRAPHY

1. **Zima, O., & R. R. Williams**
   1940. Ber. **73B**: 941.
2. **Linderstrom-Lang, K., & C. F. Jacobsen**
   1941. Jour. Biol. Chem. **137**: 443.
3. **Zima, O., K. Ritsert, & Th. Moll**
   1941. Z. physiol. Chem. **267**: 210.
4. **Michaelis, L.**
   1933. Oxidations—Reductions Potentiale. 2nd edit. Springer. Berlin.
5. **Ghosh, J. C., S. N. Raychadhuri, & S. C. Ganguli**
   1932. Jour. Indian Chem. Soc. **9**: 43.
6. **Fruton, J. S., & H. T. Clarke**
   1934. Jour. Biol. Chem. **106**: 667.
7. **Ryklan, L. R., & C. L. A. Schmidt**
   1944. University of California Publications in Physiology **8**: 257.
8. **Hopkins, F. G., & K. A. C. Elliott**
   1931. Proc. Roy. Soc. (London) **B109**: 58.
9. **Rosenbohm, A.**
   1937. Biochem. Z. **289**.
10. **Schultze, M. O., E. Stotz, & C. G. King**
    1938. Jour. Biol. Chem. **122**: 395.
11. **Bierich, R., & A. Rosenbohm**
    1935. Z. physiol. Chem. **231**: 39.
12. **Sullivan, M. X., & W. C. Hess**
    1936. Jour. Biol. Chem. **116**: 231.
13. **Schroeder, E. F., M. P. Munro, & L. Weil**
    1935. Jour. Biol. Chem. **110**: 181.
14. **Schroeder, E. F., & G. E. Woodward**
    1937. Jour. Biol. Chem. **120**: 209.
15. **Woodward, G. E., & F. E. Reinhart**
    1942. Jour. Biol. Chem. **145**: 471.
16. **Fromageot, C., E. Wookey, & P. Chaix**
    1939. Compt. rend. **209**: 1019.
17. **Smythe, C. V.**
    1942. Jour. Biol. Chem. **140**: CXXIII. 1941; **142**: 387.
18. **Fromageot, C., E. Wookey, & P. Chaix**
    1941. Enzymologia **9**: 198.
19. **Desnuelle, P., & C. Fromageot**
    1940. Enzymologia **6**: 80.
20. **Smythe, C. V., & D. Halliday**
    1942. Jour. Biol. Chem. **144**: 237.
21. **Chargaff, E., & D. B. Sprinson**
    1943. Jour. Biol. Chem. **148**: 249. 1943; **151**: 273.
22. **Binkley, F.**
    1943. Jour. Biol. Chem. **150**: 261.
23. **Bergmann, M., A. Miekeley, & E. Kann**
    1925. Z. physiol. Chem. **146**: 247.
    Bergmann, M., & K. Grafe
    1930. Z. physiol. Chem. **187**: 187.
    Bergmann, M., & D. Delis
    1927. Ann. Chem. **458**: 76.
24. **Laskowski, M., & C. Fromageot**
    1941. Jour. Biol. Chem. **140**: 663.

25. **Lawrence, J. M., & C. V. Smythe**
1943. Arch. Biochem. **2**: 225.

26. **Smythe, C. V.**
1943. Arch. Biochem. **2**: 259.

27. **Womack, M., & W. C. Rose**
1941. Jour. Biol. Chem. **121**: 403. 1937; **141**: 375.

28. **du Vigneaud, V., M. Cohn, J. P. Chandler, J. R. Schenck, & S. Simmonds**
1941. Jour. Biol. Chem. **140**: 625.

29. **Simmonds, S., M. Cohn, J. P. Chandler, & V. du Vigneaud**
1943. Jour. Biol. Chem. **149**: 512.

30. **Borek, E. & H. Waelsch**
1941. Jour. Biol. Chem. **141**: 99.

31. **Fromageot, C., & P. Desnuelle**
1942. Compt. rend. **214**: 647.

32. **Binkley, F., & V. du Vigneaud**
1942. Jour. Biol. Chem. **144**: 507.

33. **Floyd, N., & G. Medes**
1943. Arch. Biochem. **2**: 135.

34. **Blaschko, H.**
1942. Biochem. Jour. **36**: 571.

35. **Medes, G., & N. Floyd**
1943. Biochem. Jour. **36**: 836.

PUBLICATIONS

OF THE

NEW YORK ACADEMY OF SCIENCES

(LYCEUM OF NATURAL HISTORY, 1817–1876)

---

(1) The **Annals** (octavo series), established in 1823, contain the scientific contributions and reports of researches, together with the records of meetings of the Academy. The articles which comprise each volume are printed separately, each in its own cover, and are distributed immediately upon publication. The price of the separate articles depends upon their length and the number of illustrations, and may be ascertained upon application to the Executive Secretary of the Academy.

(2) The **Transactions**, reestablished as Series II in 1938, contain extended abstracts of papers presented before the regular sectional meetings of the Academy and other matters of general interest to Members, and are published monthly from November to June, inclusive.

Current numbers of the Annals and Transactions are sent free to all Members of the Academy. The Annals are sent to Honorary and Corresponding Members desiring them.

(3) The **Special Publications**, established in 1939, are issued at irregular intervals as cloth-bound volumes. The price of each volume will be advertised at time of issue.

(4) The **Memoirs** (quarto series), established in 1895, are issued at irregular intervals. It is intended that each volume shall be devoted to monographs relating to some particular department of Science. Volume I, Part 1, is devoted to Astronomical Memoirs, Volume II to Zoological Memoirs. No more parts of the Memoirs have been published to date. The price is one dollar per part.

(5) The **Scientific Survey of Porto Rico and the Virgin Islands** (octavo series), established in 1919, gives the detailed reports of the anthropological, botanical, geological, paleontological, and zoological surveys of these islands.

Subscriptions and inquiries concerning current and back numbers of any of the publications of the Academy should be addressed to

EXECUTIVE SECRETARY

The New York Academy of Sciences
Central Park West at Seventy-ninth Street
New York, N. Y.

RUDISILL AND SMITH CO., LANCASTER, PA.